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Genetic variation in the genus Leptographium with special reference to Leptographium wageneri

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GENETIC VARIATION IN THE GENUS *LEPTOGRAPHIUM* WITH SPECIAL REFERENCE TO *LEPTOGRAPHIUM WAGENERI*

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER III

ABSTRACT

GENETIC VARIATION IN THE GENUS *LEPTOGRAPHIUM* WITH SPECIAL REFERENCE TO *LEPTOGRAPHIUM WAGENERI*

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The objectives of this research were to determine genetic variation in *Leptographium* and how it corresponds to the taxonomy of the genus.

The similarity of 88 strains of 27 species of *Leptographium* was studied using enzyme electrophoresis. UGPMA cluster analysis of similarity matrices (Nei genetic identity, I) generated from data of 267 electrophoretic forms (electromorphs) of 15 enzymes showed a close correspondence between morphology and electrophoretic similarity. Strains of a species clustered at $I \geq 0.60$, but in two cases, taxa clustered at $I > 0.60$, suggesting conspecificity.

Additional isozyme studies were made of 76 isolates of *Leptographium wageneri* representing three host-specialized varieties. Of 21 enzymes tested, 10 were polymorphic, having from two to six electromorphs. Only 14 combinations (electrophoretic types) of the 29 electromorphs were found; each electrophoretic type was restricted to a single variety. Within each variety, one electrophoretic type was abundant and broadly distributed; additional types were geographically isolated or restricted. Ordination of Nei genetic distance (D) among strains revealed three discrete clusters that corresponded to the three varieties. Nei gene diversity (H) in each variety was low (0.017 to

0.040), but differentiation between varieties was high; the Nei coefficient of gene differentiation (G_{st}) for the species was 0.860.

Vegetative compatibility was tested among the same *L. wageneri* isolates by pairing auxotrophic, nitrate non-utilizing mutants on nitrate media. The development of dense hyphal growth in the zone of confrontation between complementing phenotypes indicated compatibility. Heterokaryons were recovered from hyphal tips and conidiophores of complementing pairings. Only fourteen groups of vegetatively compatible isolates (VC groups) were detected. Each contained isolates that were of similar electrophoretic types, and most had unique geographic ranges. No intervarietal complementation occurred. The indications of low genetic diversity in *Leptographium wageneri* (i.e., few electrophoretic types and VC groups), the unique geographic distributions of the electrophoretic and compatibility phenotypes, and the correlation between electrophoretic type and VC group may be due to a lack of recombination, to strong clonal selection and to founder effects.

INTRODUCTION

Leptographium Lagerb. & Melin (=*Verticicladiella* S. Hughes) is a genus containing imperfect fungi and anamorphs of some species of *Ophiostoma* H. & P. Sydow. Most *Leptographium* species are lignicolous and vectored by subcortical arthropods such as bark beetles and their associated microfauna (41). Many are agents of blue stain, and several species have been suggested to be at least weak root pathogens (1,104). The most pathogenic species of the genus, *L. wageneri* (Kendrick) Wingfield, causes black stain root disease, a unique and destructive vascular wilt disease of conifers in the western United States and British Columbia, Canada (17).

Despite the interest in some species of *Leptographium* as agents of stain and root disease and as representatives of a group of fungi with unique ecological characteristics, little has been determined of the population structure or the amount of variation within these species. The relative amount of electrophoretic differentiation separating morphologically similar species has also remained undetermined. The objective of this dissertation has been to address these deficiencies in our knowledge of this interesting and unique group of fungi.

Three studies are included as separate chapters of the dissertation. The first study determined the relatedness of strains of 27 species of *Leptographium* using UGPMA cluster analysis of electrophoretic data and discusses taxonomic implications of different levels of electrophoretic similarity. Enzyme electrophoresis, used in conjunction with morphologic studies, has been shown to be a valuable

taxonomic tool in the study of other groups of fungi (11,14,26,34,63, 95) and has been used to study variation in many organisms (4). UGPMA cluster analysis was chosen as an appropriate method for obtaining a two dimensional representation of the multi-dimensional differences in electrophoretic similarity.

The second chapter presents a more in-depth study of isozyme variation within and among three recently-described (44,45) host-specialized varieties of *Leptographium wageneri*. As only a few dimensions are needed to represent the limited electrophoretic variation that has been observed in this species, principal component analysis and three-dimensional ordination procedures were used to show relationships among strains rather than UGPMA cluster analysis. The implications of low variation and geographic distribution of variation are discussed.

A study of vegetative compatibility among strains of *Leptographium wageneri* (the third chapter) provided an alternative means of revealing and interpreting genetic differences in this species. Vegetative compatibility is the ability of hyphae of different strains to anastamose and produce cells containing nuclei of both strains. Population structure has been studied in a number of imperfect and ascomycete fungi using vegetative compatibility (3,10,12,13,51,52,54, 66,76,79). In some studies, vegetative compatibility is indicated by the development of a band of complementation between pairs of auxotrophic mutants derived from different fungus strains. In this study, complementation of nitrate non-utilizing mutants was used because of the ease with which complementary mutants can be selected on the nitrate analogue chlorate (22) and the proven usefulness of this

method (12,20,21,32,51,53,76,80,81). Results of electrophoretic and vegetative compatibility studies are compared in this chapter, and *L. wageneri* is examined as a model for understanding population structure of fungi with asexual reproduction, clonal competition, and vector dependance.

CHAPTER I

ISOZYME CHARACTERIZATION OF SPECIES IN THE GENUS *LEPTOGRAPHIUM* AND ITS TAXONOMIC IMPLICATIONS

Introduction

For many species of *Leptographium* Lagerb. & Melin, taxonomic descriptions have been vague or poorly illustrated, and type material inadequate or lacking (41). Cultures obtained from type material or other voucher specimens can be used to represent these taxa in contemporary comparisons among strains, but comparisons with such "type" cultures are also subject to error, due to changes in culture morphology that may occur over time. The loss of the teleomorphic state is common in cultures of *Ophiostoma* H. & P. Sydow (31,100), and the anamorphs and synanamorphs produced by a strain in continuous culture may become simpler in morphology. For example, studies of the species *O. clavigerum* (Robins.-Jeffr. & Davids.) Harrington (100) and *O. araucariae* (Butin) de Hoog & Scheffer (31), indicated that fresh isolates of each species can initially produce five different conidiogenous states with morphologic complexity ranging from synnematal forms, to *Leptographium*-like conidiophores, to yeast-like forms. Cultures of these species rapidly lost the ability to produce synnemata, and the yeast-like budding form eventually predominated in some sub-cultures.

Enzyme electrophoresis, used in conjunction with morphologic studies, has been shown to be a valuable taxonomic tool in the study of of several groups of fungi (11,14,26,34,62,63,95). Enzyme extracts are

separated electrophoretically in a gel matrix. Differences in the relative position of the bands showing enzyme activity are interpreted to indicate the products of alleles of one or more enzyme loci. The objective of this study was to examine electrophoretic relatedness among species and strains of the genus *Leptographium*. By determining the variation within the genus and species, the usefulness of enzyme electrophoresis for solving taxonomic problems can be evaluated.

Materials and Methods

Eighty-eight strains representing 26 *Leptographium* species were selected (Table 1). Most of the species were found on conifers, principally the Pinaceae. The strains represent about half of the described species of *Leptographium* (41), as well as unidentified and probably undescribed species. Where a number of strains of a species was available, strains were selected to represent the greatest possible geographic range.

Morphologic comparisons were made to confirm species identification. Strains were grown on water agar containing sterile sections of twigs of *Pinus resinosa* Ait. with bark removed and on 1.0 % malt extract and 1.5% agar (MEA). Cultures of *L. wageneri* (Kendr.) Wingf. were incubated at 18°C; cultures of other species were grown at room temperature. Cultures on twig medium were used for comparisons of conidiophores, conidia, and when present, perithecia and ascospores. Cultures grown on MEA were used to compare growth rates, culture appearance, characteristics of the mycelia, and in some cases, conidiophore morphology.

To obtain fresh mycelium for enzyme extraction, plugs of mycelium grown on 1.5% malt extract agar were transferred to 30 ml of liquid

TABLE 1. SPECIES AND STRAINS OF *LEPTOGRAPHIUM* AND *OPHIOSTOMA* STUDIED USING ENZYME ELECTROPHORESIS.

Species	Strain Numbers[a]	Geographic Origin	Isolated From
L. abietinum	C10 (DAOM 37981A)	British Columbia	*Picea engelmanii*
	C11	Idaho	*Pseudotsuga menziesii*
	C18 (ATCC 58568, ORF-T)	Washington	*Abies grandis*
	C42	California	*Pinus ponderosa*
	C54 (ATCC 58567, NMA-103)	New Mexico	*Pinus ponderosa*
	C172 (Gregory 1211)	Scotland	*Picea sitchensis*
	C272	New York	*Picea rubens*
	C273	New York	*Picea rubens*
L. engelmannii	C29 (RWD 971, CO 456)	Colorado	*Picea engelmannii*
L. lundbergii	C23 (NFRI 69-168)	Norway	*Pinus sylvestris*
L. procerum	C17 (NFRI 59-84/2 as *L. phycomyces*)	Norway	*Pinus sylvestris*
	C83 (ATCC 58570, IDD-102)	Idaho	*Psuedotsuga menziesii*
	C124 (NFRI 80-53/7 as *Ceratocystis polonica*)	Norway	*Picea abies*
	C323 (CBS 145.41 as *Phialocephala phycomyces*)	Unknown	wood pulp
L. pyrinum	C96 (CO 463)	Unknown	Unknown
L. serpens	C30 (CBS 141.36, from holotype)	Italy	Unknown
	C56 (PREM 45442)	Rep. South Africa	*Pinus pinaster*
	C79 (ATCC 34322)	Italy	*Pinus pinea*
	C141 (CMW 90)	Rep. South Africa	*Pinus taeda*
	C153 (Horner VPI-173)	Mississippi	*Pinus taeda*
	C169 (Horner VPI-251)	Virginia	*Pinus strobus*
	C175 (Horner VPI-256)	Virginia	*Pinus strobus*
	C297 (ATCC 42810, from *V. alacris* holotype)	Rep. South Africa	*Pinus pinaster*
	C304 (CMW 304)	Rep. South Africa	*Orthomicus erosus*
	C305 (CMW 745)	Spain	*Pinus pinaster*
	C306 (CMW 310)	Rep. South Africa	*Pinus radiata*
	C307 (CMW 382)	Rep. South Africa	*Hylastes angustatus*
L. terebrantis	C63	Massachusetts	*Pinus resinosa*
L. truncatum	C8 (PREM 45699, from paratype)	New Zealand	*Pinus strobus*
	C59 (ATCC 22735 as *L. lundbergii*)	unknown	*Pinus sylvestris*
	C167 (Juzwik 8412Lr047)	Ontario	*Pinus resinosa*

TABLE 1, continued.

L. wageneri				
var. *wageneri*	CAS-4	(ATCC 64194)	California	*Pinus monophylla*
	CAS-15	(ATCC 64195)	California	*Pinus monophylla*
var. *ponderosum*	BCL-3		British Columbia	*Pinus contorta*
	BCL-4		British Columbia	*Pinus contorta*
	CAP-36		California	*Pinus ponderosa*
	ORP-1		Oregon	*Pinus ponderosa*
var. *pseudotsugae*	BCH-1	(ATCC 42953)	British Columbia	*Tsuga heterophylla*
	CAD-55		California	*Pseudotsuga menziesii*
	COD-2	(ATCC 64191)	Colorado	*Pseudotsuga menziesii*
	IDD2	(James 80-1)	Idaho	*Pseudotsuga menziesii*
	MOD-1	(ATCC 58578)	Montana	*Pseudotsuga menziesii*
	NMD-1		New Mexico	*Pseudotsuga menziesii*
	NMD-2	(Mielke 800509)	New Mexico	*Pseudotsuga menziesii*
	ORD-Q		Oregon	*Pseudotsuga menziesii*
Leptographium sp. A	C19		Idaho	*Pseudotsuga menziesii*
	C39		Idaho	*Pseudotsuga menziesii*
Leptographium sp. E	C32		New Mexico	*Pinus ponderosa*
	C41		New Mexico	*Pinus ponderosa*
	C46	(ATCC 58571, NMP-106)	New Mexico	*Pinus ponderosa*
Leptographium sp. F	C6		California	*Pseudotsuga menziesii*
	C21	(ORD-O)	Oregon	*Pseudotsuga menziesii*
	C33	(UI 791010 as *L. abietinum*)	Idaho	*Pseudotsuga menziesii*
	C36	(Mielke 800514)	New Mexico	*Pseudotsuga menziesii*
	C40	(ATCC 58572, COD-101)	Colorado	*Pseudotsuga menziesii*
	C47		California	*Pseudotsuga menziesii*
Leptographium sp. H	C22	(ATCC 58573, IDD-101)	Idaho	*Pseudotsuga menziesii*
Leptographium sp. I	C154	(CMW 41)	Virginia	*Pinus strobus*
	C155	(CO 83-74)	Colorado	*Pinus edulis*
	C156	(CO 83-97)	Colorado	*Pinus edulis*
	C157	(CO 83-96)	Colorado	*Pinus edulis*
	C182		North Carolina	*Pinus strobus*
	C183		North Carolina	*Pinus strobus*
	C184		North Carolina	*Pinus strobus*
Leptographium sp. J	C289	(NFRC C840 as *Ophiostoma huntii*)	Alberta	*Pinus contorta*

TABLE 1, continued.

Leptographium sp. K	C173	(J. Hoffman "A")	Idaho	*Pinus edulis*
	C174	(J. Hoffman "B")	Idaho	*Pinus edulis*
Leptographium sp. L	C15	(ATCC 58566 as *L. terebrantis*, IDL-101)	Idaho	*Pinus contorta*
O. abiocarpum	C135	(RWD 494 from paratype)	unknown	*Picea engelmannii*
O. adjuncti	C119	(ATCC 34942 from holotype)	unknown	*Pinus ponderosa*
O. aureum	C88	(ATCC 16936 from holotype)	British Columbia	*Pinus contorta*
O. clavigerum	C25	(ATCC 58565 as *L. terebrantis*, BCL-101)	British Columbia	*Pinus contorta*
	C86	(CO 453)	Wyoming	*Pinus contorta*
	C187	(D. Owen 84EC (B))	California	*Pinus ponderosa*
	C291	(NFRC C837)	Alberta	*Pinus contorta*
	C295	(NFRC C1215)	British Columbia	*Pinus contorta*
O. crassivaginatum	C95	(CO 498)	Unknown	*Populus tremuloides*
O. europhioides	C129		Idaho	*Pseudotsuga menziesii*
	C274		New York	*Picea rubens*
	C290	(ATCC 16059)	Ontario	*Picea mariana*
O. huntii	C12		California	*Pinus ponderosa*
	C113	(CO 468, RWD776)	unknown	unknown
	C139		California	*Pinus ponderosa*
O. penicillatum	C5	(NFRI 1731/3)	Norway	*Picea abies*
	C7	(NFRI 1716/2)	Norway	*Picea abies*
O. robustum	C109	(CO 452)	unknown	unknown
Ophiostoma sp. M	C158		New Hampshire	*Picea rubens*
	C160		New Hampshire	*Picea rubens*

[a] Strain numbers are those used in the collection of T. C. Harrington. Abbreviations in parentheses indicate alternate strain numbers found in culture collections as follows: the American Type Culture Collection (ATCC); the Centraalbureau voor Schimmelcultures, Baarn, Netherlands (CBS); the Plant Research Institute, Dept. of Agriculture, Mycology, Ottawa, Canada (DAOM); the Norwegian Forest Research Institute, As, Norway (NFRI); the Northern Forestry Research Centre, Edmonton, Canada (NFRC); the Plant Protection Research Institute, Pretoria, South Africa (PREM); and the collections of R. W. Davidson (RWD), T. E. Hinds, U. S. Forest Service, Rocky Mountain Forest and Range Experiment Station, Fort Collins, Colorado (CO), A. D. Partridge, University of Idaho, Moscow (UI), and C. M. Wingfield, University of the Orange Free State, Bloemfontein, Republic of South Africa (CMW).

medium (20 mg malt extract plus 1.0 mg yeast extract per ml) in 125 ml Erlenmyer flasks and incubated at 18°C or room temperature. Enzymes were extracted from the mycelia of 14 day old cultures and absorbed onto paper chromatography wicks according to the protocol described in Chapter 2.

The preparation of 12% starch gels followed the method of Marty *et al.* (58). Gels were made by pouring hot, buffered starch solutions into gel forms of the design of Cardy *et al.* (15). Buffers, electrical requirements, and the amount of time required for electrophoretic separation of bands in each buffer system are described in Table 2.

Strain NMD2 of *L. wageneri* and strain C297 of *L. serpens* (Goid.) Siem. were selected as reference strains to test the evenness of electrophoretic migration across each of the gels and to calculate the relative mobility of each electrophoretic band. When gels were being loaded with sample wicks, samples of the two reference strains were placed adjacent to each other at three locations on each gel. Up to 34 sample wicks were loaded per gel.

Following electrophoresis, horizontal slices of the starch gels were stained for enzyme activity (Table 2). The distance between the electrophoretic origin and the electrophoretic band (or bands) was measured for each sample. Relative mobility (Rf) values were calculated as the ratio of the distances travelled by bands of the sample vs. reference strains and were used to determine the number of electrophoretically distinguishable forms (electromorphs) of each enzyme. Rf values from all buffer systems that gave well-resolved bands were used when determining electromorphs of an enzyme.

Secondary isozyme patterns were used in some enzymes (e.g., IDH1,

TABLE 2. ENZYMES USED IN STARCH GEL ELECTROPHORESIS OF *LEPTOGRAPHIUM* SPP., THE NUMBER OF ELECTROMORPHS DETERMINED PER ENZYME, AND BUFFERS AND STAINING PROCEDURES FAVORING RESOLUTION.

Enzyme Name (EC number)[a]	Enzyme Abbreviation[b]	Number of Electromorphs	Buffer Systems[c]	Stain Reference[d]
Aconitase (4.2.1.3)	ACO1	20	A, D, HC7	1
Aspartate aminotransferase (2.6.1.1)	AAT1	15	B2, D	1
NADH Diaphorase (1.8.1.4)	DIA1	10	A, D	1
Fumarase (4.2.1.2)	FUM1	12	A	1
Glucose-6-phosphate dehydrogenase (1.1.1.49)	G6PD1	10	B2	1
Glucosephosphate isomerase (5.3.1.9)	GPI1	24	B, D, E	1
Glutamate dehydrogenase (NADP) (1.4.1.3)	GDH1	12	B2	1
Isocitrate dehydrogenase (1.1.1.42)	IDH1	22	D, E	1
Leucine aminopeptidase (3.4.11.1)	LAP1	23	M	2
Malate dehydrogenase (1.1.1.37)	MDH1	18	D, E	1
	MDH2	20	D, E	1
Mannitol dehydrogenase (1.1.1.67)	MAN1	29	A, D, E, HC7	3
Phosphoglucomutase (5.4.2.2)	PGM1	19	A, E, HC7, M	1

TABLE 2,continued.

Superoxide dismutase (1.15.1.1)	SOD1	8	A, B2, E, HC7	4
Triose-phosphate isomerase (5.3.1.1)	TPI1	26	A, HC7	1

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a Nomenclature Committee of the International Union of Biochemistry (71).

b Multiple enzyme forms are designated in order of decreasing anodal migration.

c Buffer systems, electrical requirements, and references: **A**: pH 8.5/8.1 discontinuous TRIS citrate/lithium borate system (RW) using 50 ma constant current until wave front reaches 8 cm, Marty *et al.* (58). **B**: pH 5.7 continuous histidine citrate system using 250 V constant voltage for 4.5 hrs., Shields *et al.* (91). **B2**: pH 8.8/8.0 discontinuous TRIS citrate/sodium borate system (B) using 50 ma constant current until wave front reaches 8cm, Conkle *et al.* (19). **D**: pH 6.1 continuous morpholine citrate system using 250 V constant voltage for 5.0 hrs., Conkle *et al.* (19). **E**: buffer D with pH adjusted to 8.1 using morpholine citrate, with same voltage and run time as D. **HC7**: ph 7.0/7.0 Histidine/citrate system (HC) using 250 V constant voltage for 5.0 hrs., Marty *et al.* (58). **M**: pH 8.9 continuous TRIS borate EDTA system using 275 V constant voltage for 4.5 hrs., Micales *et al.* (62).

d 1) Marty *et al.* (58). 2) Conkle *et al.* (19). 3) Micales *et al.* (62) 4) Vallejos (103).

PGM1, and TPI1) for differentiating electromorphs of similar Rf values. Secondary isozymes or "shadowbands" are bands that result when the electrophoretic motility of a portion of the enzyme molecules is affected by changes in enzyme conformation, by binding to substrate or cofactor molecules, or by deamination. As the banding patterns that result from secondary isozymes are often highly characteristic for an allele (47), strains were considered to have different electromorphs if secondary bands were apparent in one buffer in one group of strains and in a different buffer in a different group of strains and/or if banding patterns had differences in spacing among secondary bands.

Estimates were made of the putative number of genetic loci coding for each enzyme as required for calculation of Nei genetic identity (67). Most enzymes had one set of bands, with or without shadow bands, and were considered the product of one genetic locus. If there were two sets of bands in different zones of migration in the gel, and variation in one set of bands was independant of the variation in the second set of bands (as occurred in malate dehydrogenase and NADH diaphorase) the bands of these enzymes were considered the products of two genetic loci. These estimates were checked against interpretations of the number of genetic loci coding for enzymes from a study of segregation in the related fungus *Ophiostoma nigrocarpum* (Davids.) deHoog (Zambino, unpublished) and from studies of various unrelated fungi where the genetic basis of isozyme variation is known (14,34,61, 88,90,93). Different electromorphs were assumed to represent the products of different alleles.

The routines SIMDIS and CLUSTER of the program BIOSYS-1 of Swofford and Selander (97,98) were used to calculate matrices of Nei

(67) genetic identity I (a measure of genetic relatedness) and to generate dendrograms reflecting UGPMA clustering of taxonomic units, respectively. A two step, heirarchical analysis after Swofford (98) was used to obtain the dendrograms presented in this study. In the first step, "electrophoretic types" (i.e., groups of strains that had identical electromorphs at each enzyme) were the taxonomic units analyzed in the calculation of genetic identity and dendrogram branching. In the second step, species were used as taxonomic units. Species were groups of morphologically similar strains, with or without taxonomic description as species; in one cluster with morphologic plasticity in the anamorph, the "species" was arbitrarily defined as consisting of electrophoretic types related at $I > 0.60$. To obtain allelic frequencies representative of the species, allelic frequencies from the electrophoretic types that comprised each species were averaged. In the dendrograms presented in this study, clustering and branch lengths at $I > 0.60$ were obtained from the analysis of electrophoretic types; those at $I < 0.60$ were from the analysis of species.

Results

Fifteen putative genetic loci were used for the analysis. Strains had also been screened for differences in β-glucosidase (EC 3.2.1.21) and esterase (EC 3.1.1.1) using the fluorescent stain methods of Marty *et al.* (58), but these enzymes and the less anodal form of NADH diaphorase (DIA2) were were not used in the analysis due to erratic results, the complexity of the banding patterns, poor resolution, or unequal number of bands in many samples.

The number of electromorphs for the selected enzyme loci ranged from 8 to 29 (Table 2). In the less variable enzymes, species or

groups of several related species were often found to be monomorphic, i.e., to have the same electromorph in all strains (Table 3). In contrast, many of the electromorphs of the most variable enzymes were found in only one species, and many species were polymorphic at these enzyme loci.

Cophenetic correlation (the correlation between values of relatedness in a similarity matrix versus a dendrogram) was 0.951 for the dendrogram in which taxonomic units were electrophoretic types and 0.824 for the dendrogram in which taxonomic units were species. A single difference noted between the branching patterns of the two dendrograms was the clustering of *O. abiocarpum* (Davids.) Harrington and *O. penicillatum* (Grosm.) Siem. with *O. crassivaginatum* (Griffin) Harrington in the dendrogram in which electrophoretic types were taxonomic units and with the branch containing *L. abietinum* (Peck) Wingfield in the dendrogram in which species were taxonomic units. Also, the value of I was up to 0.08 higher for some branches of the dendrogram when species were used as taxonomic units than when electrophoretic types were used as taxonomic units.

Electrophoretic relatedness among strains and species generally corresponded to their morphologic similarity. For each morphological species, there were from one to nine electrophoretic types that clustered at genetic identities of $I \geq 0.62$, suggesting close genetic relatedness. Many pairs of species that have minor morphological differences clustered at intermediate values of I (between 0.25 and 0.60). Morphological similarities of strains became increasingly obscured as the electrophoretic relatedness of the examined clusters decreased; this was particularly true of anamorph morphology. Species

TABLE 3. ENZYME ELECTROMORPHS OF SPECIES AND STRAINS OF *LEPTOGRAPHIUM* AND *OPHIOSTOMA* DETERMINED USING STARCH GEL ELECTROPHORESIS.

Species	Number of Tested Strains	Enzymes													
		ACO1[a]	AAT1	DIA1	FUM1	G6PD1	GPI1	GDH1	IDH1	LAP1	MAN1	MDH1	MDH2	PGM1	SOD1	TPI1
L. abietinum	7	J[b]	B,E	F	A,D	J	D,E,J	D	K,L,R	C,F,M	K,U	G,O	C,H	I,N	B	I
L. abietinum? C172	1	G	E	F	D	F	J	D	V	S	N	N	K	Q	B	A
L. engelmannii	1	J	B	F	D	J	D	E	L	M	U	O	H	N	B	I
L. lundbergii	1	J	J	H	E	D	V	L	M	O	B'	Q	P	O	A	L
L. procerum	4	H	I	C	C	B	I	A	C	V	D	R	F	D	F	D
L. pyrinum	1	O	A	H	H	B	R	D	M	H	V	J	F	O	D	S
L. serpens	12	A	I	F	C,G	B	B,J	H	B	K	C	B	G	F,N	A	F,V,Y
L. terebrantis	1	S	C	J	F	A	Q	B	P	E	T	J	P	K	A	T
L. truncatum	3	T	F	C,F	H	D	S	G,H	E	I	P,S	A,M	F	O	A	G,L
L. wageneri																
var. *wageneri*	2	F	L	C	F	B	K	D	S	R	G	F	O	B,E	F	M
var. *ponderosum*	4	H	M	C	F	B	K,P	D	G	R	G	F	M	E	G	H
var. *pseudotsugae*	8	H	L,M	C	F	B	K	D	G	R	G	F	M,O	B,E	F	H,O
Leptographium sp. A	2	M	C	H	G	C	X	B	M	G	J	J	N	O	A	T
Leptographium sp. E	3	D	D	C	G	B	B	D	A,B	W	B	O	F	E	A	F
Leptographium sp. F	6	C,E	D	C,G	G	B	H	D	A,B,G,Q	L,Q	B,C,E	F	A	A,C	E	F
Leptographium sp. H	1	H	O	C	G	B	K	D	G	P	F	F	O	C	A	U
Leptographium sp. I	7	L	F	C	B	D	C	E	N	D	O,R,X	E,S	Q,T	O	B	D
Leptographium sp. J	1	K	F	F	F	E	M	C	N	K	W	K	F	K	A	C
Leptographium sp. K	2	M	F	C	B	D	A	D	M	D	L	C	P	O	C	J
Leptographium sp. L	1	I	E	H	K	K	T	F	H	J	Q	A	F	O	A	X
O. abiocarpum	1	P	H	E	G	B	F	I	D	K	I	J	D	R	B	B
O. adjuncti	1	B	N	D	L	G	G	G	T	U	H	I	R	H	H	E
O. aureum	1	M	C	H	F	D	X	B	M	H	S	J	J	P	B	W
O. clavigerum	5	N,O	A	H,I,J	H	B	N,R	D	M	H,O	V	J	F,N	M,O	A,D	P,R,S
O. crassivaginatum	1	Q	G	A	J	H	W	K	W	N	C'	H	E	G	A	K
O. europhioides	3	I	I	J	F	D	S	I	G,H	B	Y	D,J	B,I,F	S	A	N,T
O. huntii	3	K	I	H	G	D	O	G	I	B	M	L	S	O	A	N
O. penicillatum	2	P	G	B	J	I	L	I	U	T	A'	P	D	J	B	D
O. robustum	1	N	A	J	H	B	N	D	M	H	V	J	F	O	A	P
Ophiostoma sp. M	2	R	K	J	I	D	U	J	J	A	Z	J	L	S	A	Q

[a] Enzyme abbreviations from Table 2.

[b] Electromorphs are designated alphabetically with electromorph A having the greatest anodal migration. In MAN1, electromorphs A', B', and C' have less anodal migration than Z.

with the greatest differences in anamorph and teleomorph morphology clustered with other species only at $I < 0.10$.

Figure 1 shows electrophoretic and morphological relatedness among strains of *L. procerum* (Kendr.) Wingfield, *L. serpens*, three varieties of *L. wageneri*, and four undescribed species. This group of species will subsequently be referred to as the "*L. serpens* cluster". They had conidia held in slime droplets, masses of young conidia were generally white, and conidiophores were produced individually from hyphae but not from stipe proliferation, even on older conidiophores. These species could be considered asexual, although there have been unconfirmed reports of perfect states occurring in two species of this cluster, i.e., *Ophiostoma wageneri* (Goheen & Cobb) Harrington (36,41) and *Ophiostoma serpens* (Goid.) von Arx (37,41). All species of the *L. serpens* cluster are from roots of conifers.

Three main clusters within the conifer pathogen *L. wageneri* corresponded to the three host-specialized varieties (clusters at $I = 0.66$ and 0.72). A strain (C22) labelled *Leptographium* sp. H, isolated from roots of *Pseudotsuga menziesii* (Mirb.) Franco., was closely related to *L. wageneri* ($I = 0.53$). *Leptographium* sp. H is weakly pathogenic to conifers (42), and does not cause the symptoms of black stain root disease found with *L. wageneri*. Although conidiophores of the two species are similar, the strain of *Leptographium* sp. H has a growth rate much slower than that of *L. wageneri*.

In addition to *L. wageneri* and *Leptographium* sp. H, there were seven other cases where a cluster of 2-3 strains or species were similar at an intermediate level (I between 0.25 and 0.60). In each case, strains of the clusters were morphologically distinguishable but

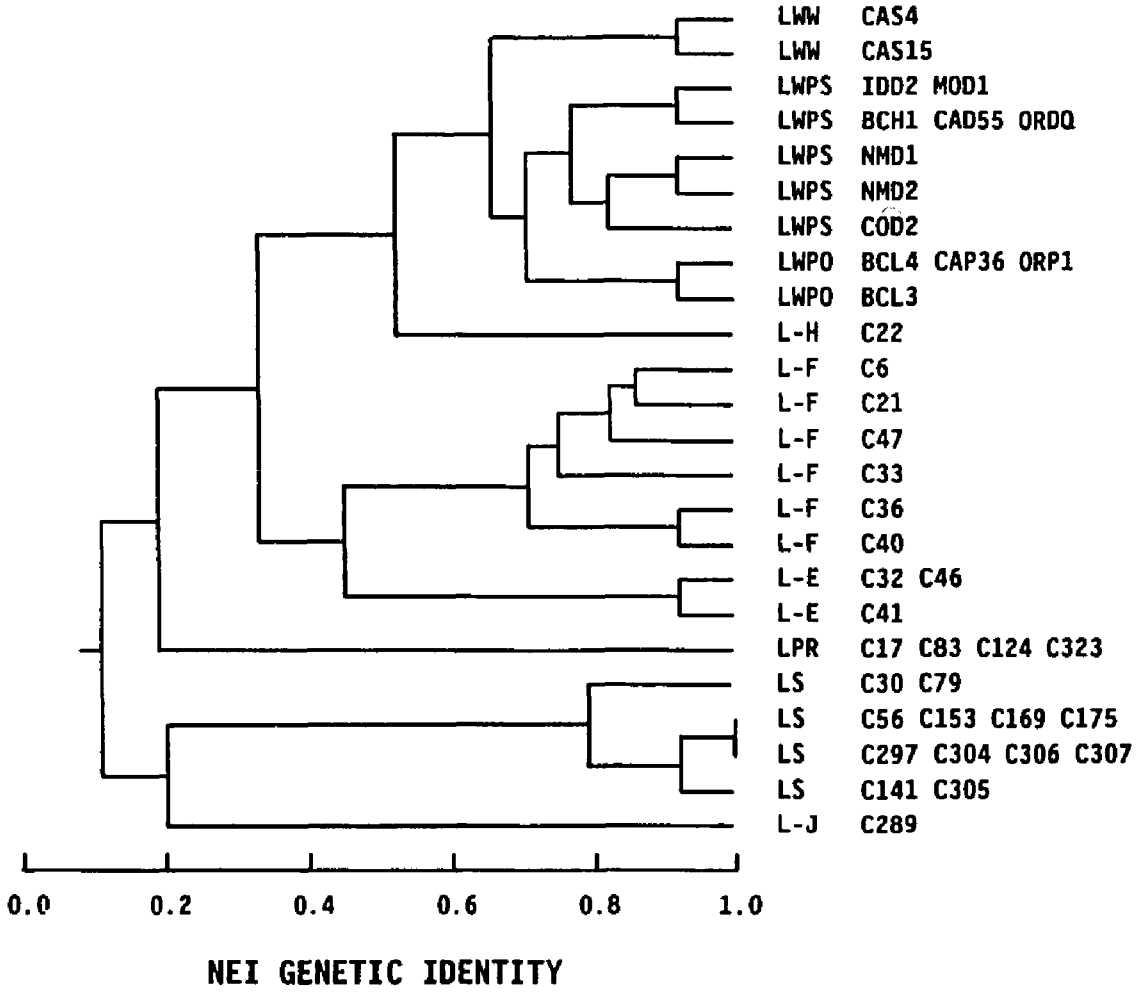

Figure 1. UGPMA cluster analysis of isozyme data from 15 putative enzyme loci showing relatedness (Nei (67) genetic identity I) among strains of five species of *Leptographium* in the *L. serpens* cluster. Species are abbreviated as follows: LPR = *L. procerum*, LS = *L. serpens*, LWPO = *L. wageneri* var. *ponderosum*, LWPS = *L. wageneri* var. *pseudotsugae*, LWW = *L. wageneri* var. *wageneri*, L-E = *Leptographium* sp. E, L-F = *Leptographium* sp. F, L-H = *Leptographium* sp. H, L-J = *Leptographium* sp. J.

had some similarities. *Leptographium* sp. E (a species found in roots of *Pinus ponderosa* Laws. (42)) and *Leptographium* F (a fungus frequently isolated from diseased roots of *Pseudotsuga menziesii* and an associate of the root-feeding bark beetle *Hylastes nigrinus* Mannerheim (42)) clustered with each other at I = 0.46 and were related to *L. wageneri* and *Leptographium* sp. H at I = 0.34. Species E and F are considered to be weak pathogens (42).

Strains of *L. procerum* from the United States and Norway were uniform for the tested enzymes, and clustered with the above species at I = 0.20. This species has a wide range of reported coniferous hosts, and has been reported as a root pathogen of pines (1).

Strains of *L. serpens*, another reported root pathogen of pines (104), were of three electrophoretic types. One consisted of two strains from Italy, including the strain C79 from the holotype; a second type consisted of a mixture of strains from the southern United States and the Republic of South Africa, including the strain C297 from the holotype of *Verticicladiella alacris* Wingfield & Marasas (later synonymized with *L. serpens* by Wingfield and Marasas (106)); the third type consisted of a strain from South Africa (C141) and a strain from Spain (C305). The three electrophoretic types clustered at I $\geq$ 0.80.

Strain C289, listed as *Leptographium* sp. J, was originally identified as *O. huntii* (Robins.-Jeffr.) deHoog & Scheffer but it was found to lack the serpentine hyphae typical of *O. huntii* (86), and conidia were more rounded than those of the three examined strains of the latter species. No ascocarps were produced. This species was somewhat related to *L. serpens* (Fig. 1), but was electrophoretically distinct from *O. huntii* (Fig. 2).

The 14 species shown in Figure 2 include seven species with *Ophiostoma* perfect states and seven species that are presumed to lack a perfect state, including the type species for the genus *Leptographium*, *L. lundbergii* Lagerb. & Melin. This cluster of species (the "*L. lundbergii* cluster") was heterogeneous in morphology. Conidia produced by different species were held in slime droplets or as semi-dry masses that ranged in color between yellow, white, grey, and light tan. Conidiophore stipes varied from single, to caespitose, to having branching proliferation. Of the species that produce *Ophiostoma* perfect states, some form perithecia that are ostiolate and necked, whereas others produce non-ostiolate perithecia. Species were isolated from trunks or from roots of pinaceous hosts.

Leptographium sp. I is an unnamed species that has similar conidiophore morphology but low relatedness to *L. serpens*. Harrington (41) has reported the width of the conidiogenous cells of strain C154 of species I to be narrower than in strains of *L. serpens*. An additional difference is the highly serpentine hyphae in strains of *L. serpens* vs. the slightly undulating but curved hyphae of species I. Lackner and Alexander (55) originally identified strain C154 as *L. serpens*, and noted its association with darkly stained roots of *Pinus strobus* L. The strains of *Leptographium* sp. I were from *Pinus strobus* and *Pinus edulis* Engelm. Strains of *Leptographium* sp. K, which clustered with *Leptographium* sp. I at I = 0.43, were obtained from roots of *Pinus edulis*.

The species *O. huntii* was described as an associate of *Dendroctonus monticola* Hopk. on bark and sapwood of *Pinus contorta* Dougl. (86) and produces ostiolate, necked perithecia and hyphae that are extremely

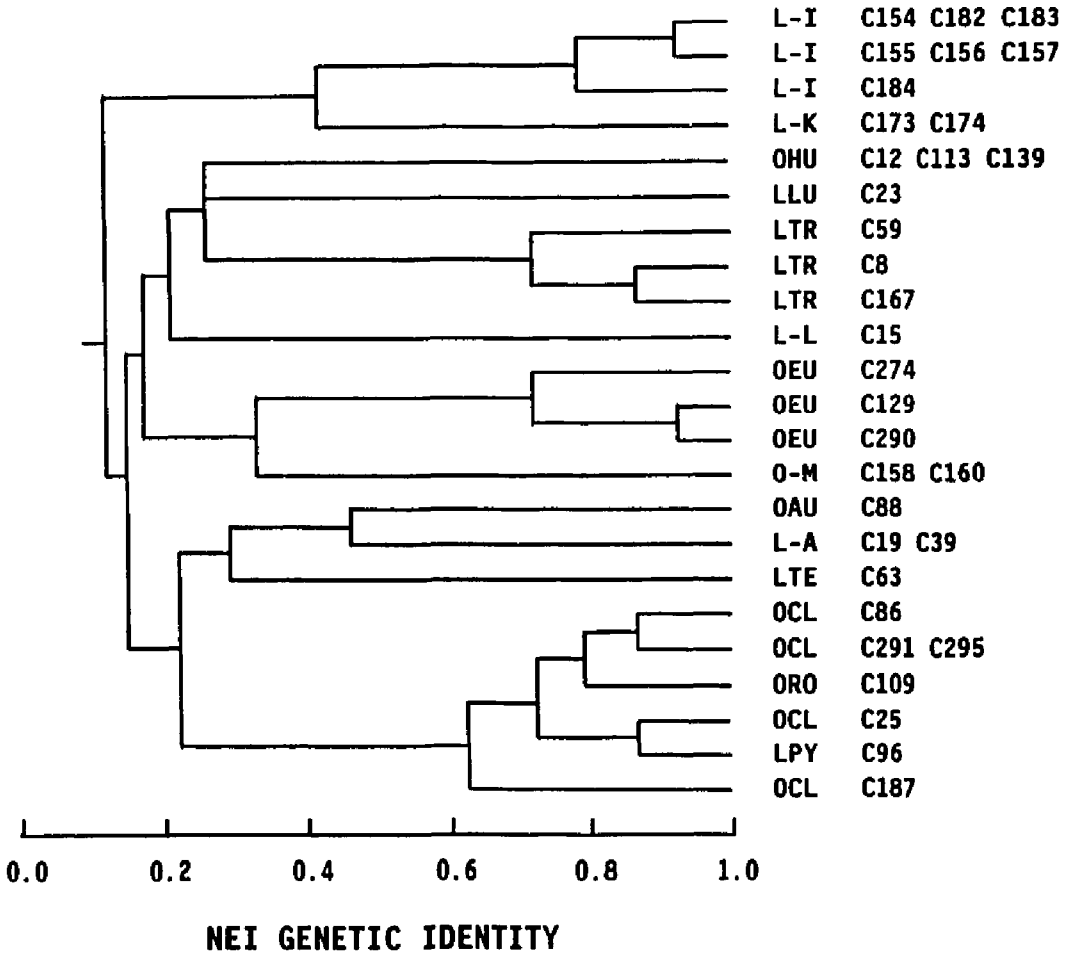

Figure 2. UGPMA cluster analysis of isozyme data from 15 putative enzyme loci showing relatedness (Nei (67) genetic identity I) among strains of 14 species of *Leptographium* and *Ophiostoma* in the *L. lundbergii* cluster. Species are abbreviated as follows: LLU = *L. lundbergii*, LPY = *L. pyrinum*, LTE = *L. terebrantis*, LTR = *L. truncatum*, L-A = *Leptographium* sp. A, L-I = *Leptographium* sp. I, L-K = *Leptographium* sp. K, L-L = *Leptographium* sp. L, OAU = *O. aureum*, OCL = *O. clavigerum*, OEU = *O. europhioides*, OHU = *O. huntii*, ORO = *O. robustum*, O-M = *Ophiostoma* sp. M.

serpentine. This fungus clustered with the asexual *L. lundbergii* and *L. truncatum* (Wingf. & Marasas) Wingf. at I = 0.27. *Leptographium lundbergii* is a common blue stain fungus on trunks of *Picea* and *Pinus* species in Europe (41,56), whereas *L. truncatum* is associated with dying roots (41,104,107). Hyphae were observed to be moderately serpentine in *L. lundbergii*, but only undulating to curved in *L. truncatum*. Conidia of the two species are somewhat similar, but basal flanges distinguish the conidia of *L. truncatum* from other species (107). Although the flanges were pronounced in conidia of strain C8, a strain isolated from a paratype specimen, they were difficult to discern in the strain C59. Strains C59 and C23 had been received as *L. lundbergii*, but C23 more closely matched the description of *L. lundbergii* by Lagerberg *et al.* (56).

Several cases have been mentioned where strains labelled as the same species were found to cluster to different portions of the dendrogram. In each case, a reexamination of the morphologies of the strains revealed that one or another of the strains had been misidentified. Strains C15 and C25, here labelled as *Leptographium* sp. L and *O. clavigerum*, respectively, had likewise been misidentified. Strain C15 was isolated from *Pinus contorta* and may be of the same taxon reported by Mielke (64) as *L. penicillatum* (see Harrington (41)). Later, Harrington and Cobb (42) reported the pathogenicity of C15 and C25 under the name *L. terebrantis* Barras & Perry.

Strains C15, C25, and C63 had been chosen for the present study to represent variation within *L. terebrantis*. After the electrophoretic analysis showed little genetic similarity among the strains, further morphologic comparisons were made among 21 strains of *L. terebrantis*

the collection of T. C. Harrington. Strain C63 and most of the other strains were typical of Barras and Perry's (7) description of *L. terebrantis*, whereas strain C25 was found to have conidiophores that fit within the range of morphologies observed in *O. clavigerum*. Strain C15 differed in conidiophore and mycelial characteristics from the desciption of *L. terebrantis*, *O. penicillatum*, and all other examined species.

Strains labelled as *O. europhioides* (Wright & Cain) H. Solheim and *as Ophiostoma* sp. M were related at I = 0.34 and produced ostiolate perithecia and ascospores of similar dimensions. Clustered, branched conidiophores typical of the species (109) were abundant in strains of *O. europhioides*, but the strains of *Ophiostoma* sp. M lacked a *Leptographium* anamorph. *Ophiostoma* sp. M also lacked the branched perithecial necks found with strains of *O. europhioides* (109). The strains of *Ophiostoma* sp. M were also compared with the description of *O. piceaperdum* (Rumb.) von Arx, as *O. europhioides* is similar to *O. piceaperdum* and is considered a synonym by Upadhyay (101). As with *O. europhioides*, *O. piceaperdum* produces abundant conidiophores (89). Rumbold (89) did not specifically mention neck branching in *O. piceaperdum*.

A culture (C88) from the holotype of *O. aureum* (Robins.-Jeffr. & Davids.) Harrington, a species associated with the stems of beetle-infested pines (85), clustered at I = 0.47 with two strains of similar morphology that had been isolated from *Pseudotsuga menziesii* attacked by *Dendroctonus pseudotsugae* Hopk. and that have been designated *Leptographium* sp. A (41). The two species produced conidiophores that were similar in size, shape, and the arrangement of metulae. Masses of

conidia were yellow in both species, but conidium size was up to twice as long in *O. aureum* as in *Leptographium* sp. A. Although perithecia of *O. aureum* were not produced in the current study, they reportedly (85) lack necks and are astomatous.

Strain C63 of *L. terebrantis* had conidia, conidiophores, and mycelia that differed greatly from *O. aureum* with which it clustered at I = 0.30. *L. terebrantis* also differed greatly from strains in the *O. clavigerum* cluster.

Ophiostoma clavigerum, *O. robustum* (Robins.-Jeffr. & Davids.) Harrington, and *L. pyrinum* Davids. clustered with one another at I ≥ 0.63, indicating close relatedness. These three species are found in stems of pines attacked by bark beetles (30,85). Anamorph morphologies of the strains were consistent with their respective species descriptions (30,85). *Ophiostoma clavigerum* produced clavate conidia of two sizes, some extremely long and multicellular, others small and unicellular. *Ophiostoma robustum* produced rounded to oval conidia of several sizes. In both of these species, many conidia had thick cell walls. *Leptographium pyrinum* produced pear-shaped conidia with unthickened cell walls. Some strains of *O. clavigerum* produced synnematal structures in addition to mononematous conidiophores typical of *Leptographium*, a salient feature of Upadhyay and Kendrick's genus *Graphiocladiella* (101). The production of the larger, synnematal structures was not found in all strains of *O. clavigerum*, however. Only mononematous conidiophores were found in *O. robustum* and *L. pyrinum*. There was some similarity between *O. aureum*, *Leptographium* sp. A, and *O. clavigerum* in the branching patterns of the metulae of the conidiophores, and the teleomorphs of *O. aureum*, *O. robustum*, and

O. clavigerum have been reported to be similar if not identical (85).

Figure 3 shows the relatedness among the remaining species used in the study, the *L. serpens* cluster, and the *L. lundbergii* cluster. Strains of *L. abietinum* (Peck) Wingf. were from various pinaceous hosts and geographic origins. The strain C29, received from Davidson's collection as *L. engelmannii* Davids. and isolated from *Picea engelmannii* Parry in Colorado, was similar in morphology to the other strains of *L. abietinum* and was electrophoretically identical to strain C272 from *Picea rubens* Sarg. in New York. The strain C172, from diseased roots of *Picea sitchensis* (Bong.) Carr. in Scotland (38), had been previously identified as *L. abietinum* by Harrington, but this strain had several morphologic differences that distinguished it from strains of *L. abietinum*. Conidia of the two taxa were similar, and conidiophores of strain C172 were similar to those of the other strains in terminal branching patterns, but additional thin branches originated near the base of the conidiophores of this strain that resembled hyphae in width and pigmentation, and ended with a single conidiogenous cell. All other strains of *L. abietinum* lacked such branches. Growth of C172 was slower than strains of *L. abietinum* on MEA and was markedly zonate on twig medium. This strain was related to typical strains of *L. abietinum* at $I = 0.31$, a level much lower than the level $I > 0.60$ that represented the minimum value for within-species relatedness in other species.

Strains representing the species *O. penicillatum* and *O. abiocarpum* were related at $I = 0.27$. The conidiophores and conidia of strains C5 and C7 were typical of the anamorph of *O. penicillatum*, but strain C135 of *O. abiocarpum* did not produce conidiophores in culture. Davidson

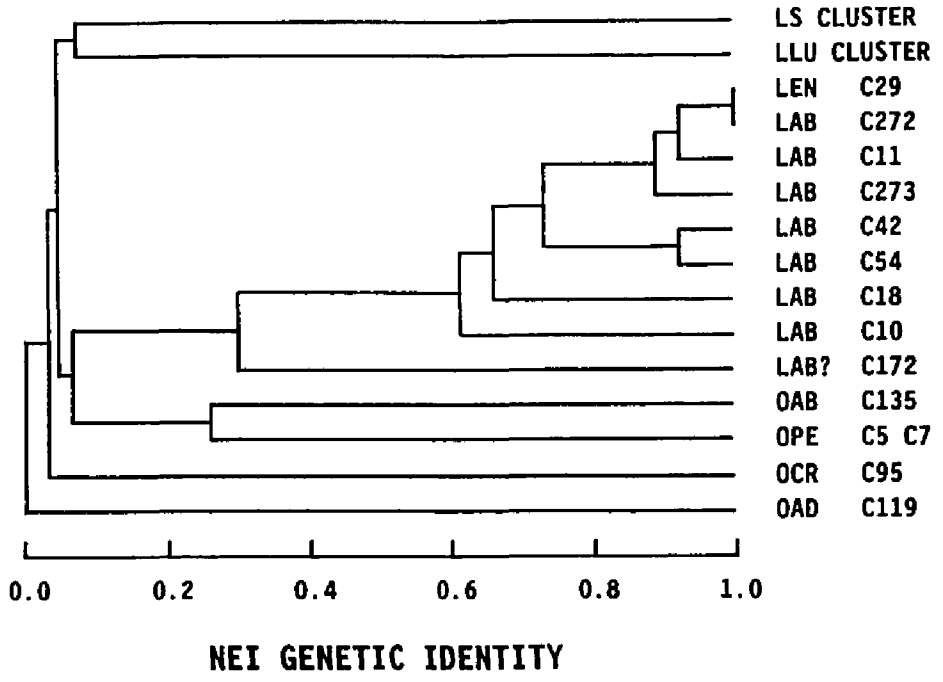

Figure 3. UGPMA cluster analysis of isozyme data from 15 putative enzyme loci showing relatedness (Nei's (67) genetic identity I) among strains of 6 species of *Leptographium* and *Ophiostoma* and the *L. serpens* and *L. lundbergii* clusters from Figs. 1 and 2. Species and clusters are abbreviated as follows: LAB = *L. abietinum*, LEN = *L. engelmannii*, OAB = *O. abiocarpum*, OAD = *O. adjuncti*, OCR = *O. crassivaginatum*, OPE = *O. penicillatum*, LS Cluster = *L. serpens* cluster from Fig. 1, LLU Cluster = *L. lundbergii* cluster from Fig. 2.

(29) reported the lack of a *Leptographium* state to be typical in *O. abiocarpum* and questioned whether *Leptographium*-like conidiophores found near ascocarps of this species were produced by the same fungus, but Upadhyay (101) confirmed the anamorph-teleomorph connection. Ascocarps were not produced by *O. penicillatum* or *O. abiocarpum* in this study.

The species used in this study represented four distinct ascospore morphologies. In the *L. lundbergii* cluster, ascospores have gelatinous sheaths and appeared hat-shaped in side view. Ascospores of *O. penicillatum* and *O. abiocarpum* are allantoid (29,93), and the gelatinous ascospore sheaths are variously reported as lacking (29) or as being of variable thickness (93). *Ophiostoma adjuncti* (Davids.) Harrington, a species associated with the bark beetle *Dendroctonus adjunctus* Blandford attacking *Pinus ponderosa*, produces sheathed ascospores that appear rectangular or pillow-shaped (30). *Ophiostoma crassivaginatum* has falcate ascospores and occurs primarily on hardwoods (41,101). Relatedness was very low ($I \leq 0.07$, Fig. 3) among the four branches that represent these ascospore morphologies, with *O. adjuncti* having the lowest relatedness to the other branches.

Discussion

Isozyme analysis was useful for showing relationships among groups of strains, infraspecific taxa, species, and species clusters of the genus *Leptographium*. The value of $I = 0.60$ may be useful in delimiting species. Clustering at greater levels of relatedness can indicate the need for taxa to be synonymized (e.g., *L. abietinum* and *L. engelmannii*); relatedness at less than this level indicates the need for strains differing slightly in morphology to be described as distinct

taxa (e.g., *Leptographium* sp. I from *L. serpens*).

The validity of this "genetic yardstick" is supported by the lack of correlation between clustering and geographic origin or host origin, although these factors could affect strain clustering at greater levels of relatedness. To the extent that isozyme studies of other genera can be compared, the value of I = 0.60 is also supported by its general agreement with the minimum relatedness of approximately I = 0.50 for the ascomycete *Cryphonectria cubenses* (Bruner) Hodges (63).

In the current study, strains of *O. clavigerum*, *O. robustum*, and *L. pyrinum* (Fig. 2) formed a distinct cluster and can be considered conspecific in terms of relatedness. In light of the inherent variability in anamorph morphology noted in *O. clavigerum* (100,85) and the lack of differences in teleomorph characteristics between *O. clavigerum* and *O. robustum* noted in the diagnoses by Robbinson-Jeffrey and Davidson (85), it is likely that the three taxa are morphologic variants of the same species and should probably be synonymized. However, more strains and holotype material should be examined. It is noteworthy that the morphologically similar *O. aureum* did not cluster closely with *O. clavigerum*.

A suggestion by Harrington (41) that *L. engelmannii* and *L. abietinum* may be conspecific is supported by close clustering of all strains of the two taxa except the atypical C172. Davidson's (27) description of *L. engelmannii* closely resembles *L. abietinum*, and since the diagnosis of *L. engelmannii* did not include a comparison with *L. abietinum*, Davidson may have been unaware of the resemblance between his fungus and the earlier described *L. abietinum*. Strain C29 from the collection of Davidson is the only known strain of *L. engelmannii* (41),

but its relationship to the type specimen is uncertain.

Taxonomic units that were morphologically similar but distinguishable and that had relatedness of I between 0.25 and 0.60 can be interpreted as distinct but closely related species, i.e., sib-species. Davidson (29) first recognized *O. abiocarpum* as distinct from its European sib-species *O. penicillatum* by its lack or rarity of *Leptographium* conidiophores, and these species had relatedness of I = 0.27. If more strains could be obtained for further and more detailed morphologic characterization, a number of taxa of intermediate relatedness could potentially be described as new species. For example, in this study, strains of *Ophiostoma* sp. M differed from strains of its sib-species *O. europhioides* (and, presumably, from the related species *O. piceaperdum*, not tested in this study) by the lack of conidiophores and the unbranched perithecial necks in *Ophiostoma* sp. M (109). Similarly, strain C172 was distinct from from all other strains of *L. abietinum* by its production of thin conidiogenous branches from the base of the conidiophore stipe and may be a sib-species of the latter fungus.

Electophoretic results of this study support the decision by Wingfield and Marasas (106) to synomymize *Verticicladiella alacris* with *L. serpens*, and demonstrate the utility of this technique in identifying older or otherwise atypical cultures. *Leptographium serpens* was descibed over 50 years ago from a strain attacking pines in Italy. The original material used to describe this species has been lost, but strain C30 from the type specimen is available. Morphology of this culture differs from recent cultures of *L. serpens* in having shorter conidia, slower growth, less serpentine hyphae, and unusual side

branches along the main stipe of the conidiophore (41,106). In contrast to culture morphology, electrphoretic characters were apparently unaffected by the age of the culture.

Although branching at the low extremes of relatedness may be subject to some error, the low relatedness among species representing different ascospore morphologies (72) (i.e., *O. adjuncti*, *O. crassivaginatum*, *O. penicillatum/O. abiocarpum*, and *L. lundbergii* cluster) provided some justification for the subdivision of the genus *Ophiostoma* into sections or groups along these lines. However, the fact that *O. adjuncti* of Olchowecki and Reid's (72) Ips group had lower relatedness than *O. crassivaginatum* to other *Ophiostoma* species suggests that many of the species of Upadhyay's (101,102) genus *Ceratocystiopsis* can be accomodated in *Ophiostoma*, as suggested by de Hoog (31) and Harrington (40,41).

In conclusion, enzyme electrophoresis has been shown to be a valuable tool for clarifying the taxonomy of the genus *Leptographium*. The results of the current study suggest the further use of this method for determining the extent to which morphologic variation is due to infra-specific variation versus differences at the species level among as yet unrecognized and undescribed taxa of poorly understood species complexes.

CHAPTER II

ISOZYME VARIATION WITHIN AND AMONG HOST-SPECIALIZED VARIETIES OF *LEPTOGRAPHIUM WAGENERI*

Introduction

Leptographium wageneri (Kendrick) Wingfield is a dematiaceous hyphomycete that causes a unique and serious wilt-type disease of conifers in western North America, black-stain root disease (17). Three physiologically and morphologically distinct variants of the fungus are specialized to different host species (43). These host-specialized variants have recently been recognized as distinct taxonomic varieties (44,45). *Leptographium wageneri* var. *wageneri* is pathogenic to pinyons; variety *ponderosum* (Harrington & Cobb) Harrington & Cobb is primarily specialized to western hard pines and rarely attacks white pines and hemlocks; variety *pseudotsugae* Harrington & Cobb causes black stain in Douglas-fir and has also been isolated from western hemlock (43).

Cultures of the three varieties grown on agar medium differ slightly in growth rates at 25°C, in pigmentation, in the relative production of conidiophores, and in width of conidiophore stipe apices (45). There has been only a single report of a teleomorph in this species: *Ophiostoma wageneri* (Goheen & Cobb) Harrington was described from perithecia in beetle galleries in roots of a diseased ponderosa pine (36).

Although taxonomy is based on observable differences in morphology, biochemical techniques have become increasingly important as an

adjunct to traditional morphologic studies. Electrophoresis of soluble enzymes is an indirect method of determining differences at enzyme loci and has recently been used to delineate taxa that are morphologically similar or variable (11,26,63). Isozyme frequency data have also been useful for identifying factors that affect genetic variation in populations or species. For example, the lack of sexual reproduction (57,98), environmental homogeneity (95), and founder effects (95) have all been correlated with low electrophoretic variability.

In a recent study, Otrosina and Cobb (73) used starch gel electrophoresis of ten enzymes to study isozyme variation among 26 isolates representing the three varieties of *L. wageneri*. Their data suggested low genetic variation in the species, with seven of the ten enzymes having only one electromorphic form. The data from the three polymorphic enzymes supported the concept of three taxonomic varieties.

Variation in *Leptographium wageneri* was further examined in the current study, but more isolates and enzymes were used. The validity of the current concept of three taxonomic varieties was separately tested 1) by using distance matrix methods for individual isolates and for varieties; 2) by calculation of the amount of differentiation between varieties compared to the diversity in the species as a whole; and 3) by noting the occurrence of enzyme electromorphs and their combinations in different varieties. A second objective was to utilize the amount and distribution of electrophoretic variation in the species as indicators of the prevalence of sexual reproduction in *Leptographium wageneri* in nature.

Materials and Methods

Seventy-six isolates of *Leptographium wageneri* were selected to broadly represent the range of hosts and geographic areas where the fungus has been reported (Fig. 4, Table 4). Each isolate was obtained from a different infection center.

To obtain fresh mycelium for enzyme extraction, pieces of culture grown on 1.5% malt extract agar were added to 30 ml of liquid medium (20 mg malt extract plus 1.0 mg yeast extract per ml) in 125-ml Erlenmyer flasks. Isolates were grown in still culture at 18°C for 14 days. Mycelial mats were vacuum filtered to remove excess medium, placed in pre-chilled mortars, frozen with liquid nitrogen, and ground to a fine powder. Enzymes were extracted by further grinding with 0.75 ml of chilled extraction buffer prepared by mixing 0.2 M Na_2PO_4 (25 ml), glycerol (25 ml), deionized water (50 ml), lyophilized bovine serum albumin (1.0 g) and disodium EDTA (0.17 g) and adjusting the pH to 7.1. Crude enzyme extracts were absorbed through miracloth onto 4 x 12 mm wicks of Whatman No. 3MM chromatography paper and ultrafrozen at -80°C.

Buffer systems used in electrophoresis and staining procedures were those of Conkle *et al.* (19), Marty *et al.* (58), Micales *et al.* (62), and Shields *et al.* (91) and are listed in Table 5 along with names and abbreviations of each of the putative enzyme loci used in this study. Twelve percent starch gels were prepared one day prior to electrophoresis according to the microwave methods of Marty *et al.* (58). The heated starch mixtures were poured into gel trays designed to eliminate the need for sponge or cloth electrode wicks (15).

Gels were loaded with thirty wicks, each representing a different

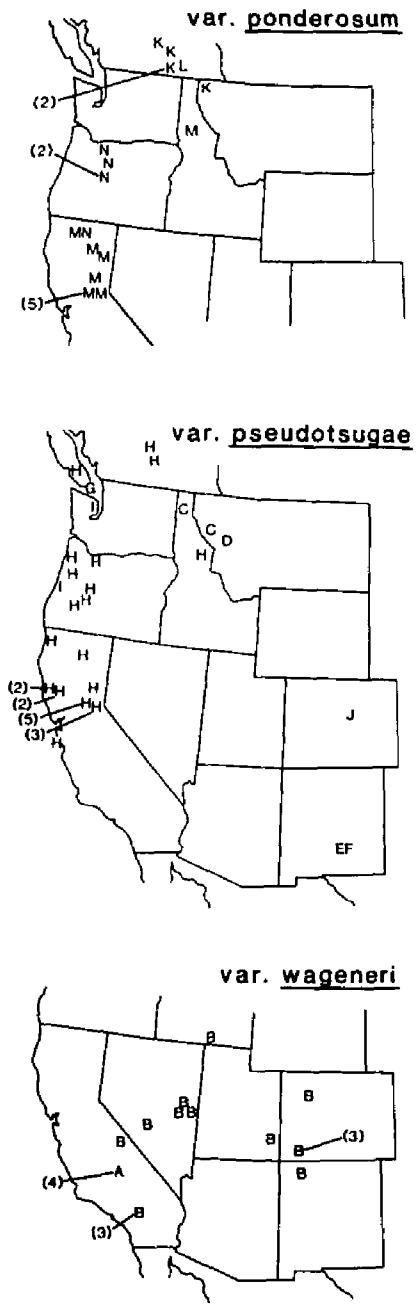

Figure 4. Geographic distribution of electrophoretic types of three varieties of *Leptographium wageneri*. Top = var. *ponderosum*, middle = var. *pseudotsugae*, bottom = var. *wageneri*. The position of each letter represents the approximate origin of one isolate unless otherwise indicated.

TABLE 4. ISOLATES OF *LEPTOGRAPHIUM WAGENERI* ARRANGED BY VARIETY, ELECTROPHORETIC TYPE, AND HOST OF ORIGIN.

Variety	Type	Host	Isolates[a]
var. *wageneri*	A	*Pinus monophylla*	CAS4(ATCC64194), CAS5, CAS7, CAS9
	B	*Pinus edulis*	COE1(ATCC58576), COE2, COE6, COEN, IDE1, NME1(ATCC58579), UTE1
		Pinus monophylla	CAS1(ATCC64193), CAS2, CAS3, CAS15(ATCC64195), NES1(ATCC64192), NES2, NES3, NES4
var. *pseudotsugae*	C	*Pseudotsuga menziesii*	IDD2, MOD22
	D	*Pseudotsuga menziesii*	MOD1(ATCC58578)
	E	*Pseudotsuga menziesii*	NMD1
	F	*Pseudotsuga menziesii*	NMD2
	G	*Tsuga heterophylla*	BCH1(ATCC42953)
	H	*Pseudotsuga menziesii*	BCD1(ATCC58574), BCD11, BCDJ, CAD1, CAD2, CAD5, CAD6, CAD18(ATCC64196), CAD19, CAD22, CAD27, CAD30, CAD31, CAD32, CAD40, CAD55, CAD56, CADF, CADX, IDD1, ORD1, ORD2, ORD3, ORD4, ORD5, ORDP
	I	*Pseudotsuga menziesii*	ORDQ, WADU
	J	*Pseudotsuga menziesii*	COD2(ATCC64191)
var. *ponderosum*	K	*Pinus contorta*	BCL1(ATCC42954), BCL2, BCL4
		Pinus monticola	BCW1
		Pinus strobus	MOW2

TABLE 4, continued.

L	*Pinus contorta*	BCL3
M	*Pinus jeffreyi*	CAJ1, CAJ3
	Pinus ponderosa	CAP3, CAP19(ATCC58575), CAP36, CAPC, CAPD, CAPH, CAPI, CAPW, IDP1(ATCC58577)
	Tsuga mertensiana	ORMS(ATCC58581)
N	*Pinus contorta*	ORL1
	Pinus ponderosa	CAPY, ORP1
	Tsuga heterophylla	ORH1(ATCC58580)

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[a] Culture numbers are those used in the collection of T. C. Harrington. The first two letters designate the state or province of origin. Numbers in parentheses are those of the American Type Culture Collection.

isolate. Samples representing each electromorph of *L. wageneri* and examples of other fungi in *Leptographium*, *Ophiostoma*, and *Ceratocystis* were included as references. Wicks were removed after 10-15 minutes of electrophoresis at the voltages specified in Table 5.

After electrophoresis, up to five slices per gel were stained for enzyme activity. A cathodal slice was included along with an anodal slice in stains for enzymes that were determined to have electromorphs with cathodal migration in initial tests (e.g., MDH2).

Several staining procedures had the potential to detect activity of more than one enzyme. In each case, the identification of electrophoretic bands was determined by staining other slices of the same gel using staining procedures specific for activity of the other enzymes that may have been detected. Sets of bands on subsequent slices having the same patterns of electrophoretic motility were assumed to represent activity of the same enzyme system. Bands on gel slices stained for the two MDH systems were compared in this manner. Slices stained for DIA and MNR were compared with each other and with slices stained for glutathione reductase (EC 1.6.4.2) for the presence of shared banding patterns.

Enzymes were selected that had well-resolved, well-stained bands and an equal number of bands in all isolates (Fig. 5, Table 5). Electromorphs were determined for each isolate after a comparison of its banding pattern in one or more gels of each of the buffer systems used for the enzyme. Estimates of relative electrophoretic differences among isolates, i.e., "electrophoretic distances", were calculated using Nei (67) genetic distance D; distance estimates were arranged in matrix form. Because of minimal loss of information and the ability to

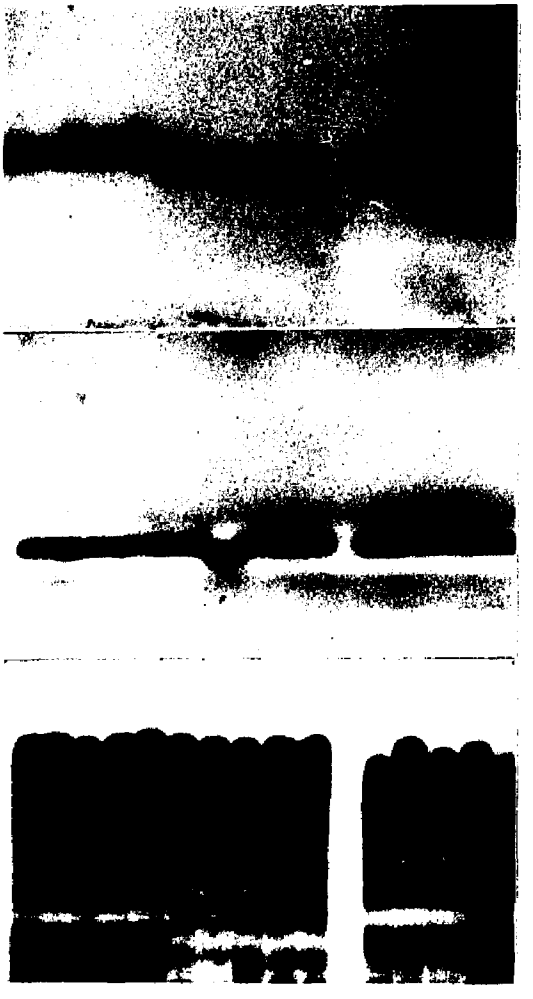

Figure 5. Electromorphs of *L. wageneri* for aconitase (top), glucose-phosphate isomerase (middle), and superoxide dismutase (destained bands, bottom). From left to right, the lanes of each gel are as follows: lanes 1-5 are isolates CAS1, CAS3, CAS4, CAS5, and CAS7 of var. *wageneri*; lanes 6-10 are isolates BCL1, BCL3, BCL4, BCW1, and CAJ1 of var. *ponderosum*; lanes 11-15 are isolates BCD1, BCD11, BCH1, CAD18, and CAD31 of var. *pseudotsugae*.

TABLE 5. ENZYMES USED IN STARCH GEL ELECTROPHORESIS OF *LEPTOGRAPHIUM WAGENERI*, THE NUMBER OF ELECTROMORPHS DETERMINED PER ENZYME, AND BUFFERS AND STAINING PROCEDURES FAVORING RESOLUTION.

Enzyme Name (EC number)[a]	Enzyme Abbreviation[b]	Number of Electromorphs	Buffer Systems[c]	Stain Reference[d]
Aconitase (4.2.1.3)	ACO1	2	A, HC7	2
Aspartate aminotransferase (2.6.1.1)	AAT1	3	B2, D	2
Catalase (1.11.1.6)	CAT1	1	B2	1
NADH Diaphorase (1.8.1.4)	DIA1	1	A	2
	DIA2	5	A, D	2
Esterase (3.1.1.1)	EST1	3	D	3
Fumarase (4.2.1.2)	FUM1	1	A	2
Glucose-6-phosphate dehydrogenase (1.1.1.49)	G6PD1	1	A, B2	2
Glucosephosphate isomerase (5.3.1.9)	GPI1	2	A, B	1
β-Glucosidase (3.2.1.21)	β-GLU1	6	B, D	2
Glutamate dehydrogenase NADP (1.4.1.3)	GDH1	1	B, B2	2
Isocitrate dehydrogenase (1.1.1.42)	IDH1	2	E	2
Leucine aminopeptidase (3.4.11.1)	LAP1	1	M	1

TABLE 5, continued.

Malate dehydrogenase (1.1.1.37)	MDH1	1	D, E	2
	MDH2	1	D, E	2
Menadione reductase (1.6.99.2)	MNR1	1	A	1
	MNR2	1	A	1
Peptidase (3.4.13)	PEP1	1	A	2
Phosphoglucomutase (5.4.2.2)	PGM1	2	B, D	2
Superoxide dismutase (1.15.1.1)	SOD1	2	HC7	4
Triose-phosphate isomerase (5.3.1.1)	TPI1	2	A	2

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[a] Nomenclature Committee of the International Union of Biochemistry (71).

[b] Multiple enzyme forms are designated in order of decreasing anodal migration.

[c] Buffer systems, electrical requirements, and references: **A**: pH 8.5/8.1 discontinuous TRIS citrate/lithium borate system (RW) using 50 ma constant current until wave front reaches 8 cm, Marty *et al.* (58). **B**: pH 5.7 continuous histidine citrate system using 250 V constant voltage for 4.5 hrs., Shields *et al.* (91). **B2**: pH 8.8/8.0 discontinuous TRIS citrate/sodium borate system (B) using 50 ma constant current until wave front reaches 8cm, Conkle *et al.* (19). **D**: pH 6.1 continuous morpholine citrate system using 250 V constant voltage for 5.0 hrs., Conkle *et al.* (19). **E**: buffer D with pH adjusted to 8.1 using morpholine citrate, with same voltage and run time as D. **HC7**: ph 7.0/7.0 Histidine/citrate system (HC) using 250 V constant voltage for 5.0 hrs., Marty *et al.* (58). **M**: pH 8.9 continuous TRIS borate EDTA system using 275 V constant voltage for 4.5 hrs., Micales *et al.* (62).

[d] 1) Conkle *et al.* (19). 2) Marty *et al.* (58). 3) Fluorescent esterase stain of Marty *et al.* (58). 4) Destained SOD bands on blue background; 6-Phosphogluconic dehydrogenase stain of Marty *et al.* (58).

use data without *a priori* classification (16), ordination of the matrix data by principal coordinate analysis was used to show distance relationships among all isolates, among isolates of var. *pseudotsugae*, and among isolates of var. *ponderosum* but was not used to show relationships among isolates of the less variable var. *wageneri*. For each analysis, a scatter plot was constructed using the first three principle coordinates.

Nei (67) genetic distance was also calculated between each pair of varieties, and estimates of Nei (68) gene diversity H within and between varieties and Nei (68,70) gene differentiation G_{ST} and R_{ST} within *L. wageneri* at the level of variety were obtained.

Attempts were made to induce the sexual state of *L. wageneri* in order to investigate the genetic inheritance of isozyme variation in this fungus. Isolates representing each of 14 electrophoretic types (i.e., groups of isolates with detectable electrophoretic differences, Table 6) were paired in all combinations on water agar containing sterile sections of twigs of *Pinus resinosa* Ait., 1.0 ppm thiamine hydrochloride, 0.75 ppm pyridoxine hydrochloride, and 0.05 ppm biotin. Plates were incubated at 18°C and examined for the production of perithecia and protoperithecia at various intervals.

Results

Each of the enzymes from Table 5 yielded dark, well-resolved patterns and showed electrophoretic variation between the isolates of *L. wageneri* and distantly related fungi. In most of the enzymes, only one major enzyme form was indicated, but there were two electrophoretic forms of DIA, MDH and MNR, giving a total of 21 scorable enzymes. Eleven of these had only one detectable electromorph in all isolates of

L. wageneri. The number of electromorphs in the ten polymorphic enzymes ranged from two to six (Tables 5 and 6).

Although variation in most enzymes was easy to determine (Fig. 5), the results from four enzymes needed particular care in interpretation and determination of electromorphs. The enzyme GDH1 was apparently monomorphic, but had a great deal of variability in activity and darkness of staining between isolates. No GDH1 activity was detected for isolates CAS9 and CAP19 despite repeated attempts. Although the lack of staining could be interpreted as representing one or possibly two null alleles, it has been treated as missing data. Because of this, data from this enzyme could not be used in calculations of Nei genetic distance and Nei gene diversity.

A triple banding pattern was found in the enzyme G6PD1. The band with the least anodal migration was broader and less resolved than the other two bands in all isolates, but in certain isolates the band was particularly diffuse and slower in migration. These observed differences between isolates in resolution and migration of the third band were eliminated when isolates were grown on a medium with a high glucose content (unpublished data). The triple-banded patterns may therefore indicate the product of one monomorphic locus. In contrast, the triple banding pattern has been interpreted by Otrosina and Cobb (73) to indicate one monomorphic and one polymorphic locus.

The enzyme β-GLU1 had five electromorphs that resolved into sharply-defined fluorescent bands. However, four isolates of var. *ponderosum* had minimal activity of this enzyme, detectable only as a diffuse background fluorescence (Table 6). Since the four isolates were from the same geographic area (Fig. 4, top) and were identical for

TABLE 6. ELECTROMORPHS OF THE 14 ELECTROPHORETIC TYPES FOUND FOR 76 ISOLATES OF *LEPTOGRAPHIUM WAGENERI*. ENZYME ELECTROMORPHS[a] ARE DISPLAYED IN ROWS, ELECTROPHORETIC TYPES IN COLUMNS ARRANGED ACCORDING TO VARIETY.

	Electrophoretic Types[b]													
	var. *wageneri*		var. *pseudotsugae*								var. *ponderosum*			
Enzymes	A	B	C	D	E	F	G	H	I	J	K	L	M	N
ACO1	a[c]	a	b	b	b	b	b	b	b	b	b	b	b	b
AAT1	a	a	a	a	a	a	b	b	b	b	c	c	c	c
DIA2	d	d	e	c	e	c	e	e	e	e	a	a	b	b
EST1	a	a	b	b	b	b	c	b	b	b	b	b	b	b
GPI1	a	a	a	a	a	a	a	a	a	a	a	b	a	a
β-GLU1	c	c	a	a	a	d	a	a	e	a	b	b	b	n[d]
IDH1	b	b	a	a	a	a	a	a	a	a	a	a	a	a
PGM1	a	b	a	a	b	b	a	a	a	b	b	b	b	b
SOD1	b	b	a	a	a	a	a	a	a	a	b	b	b	b
TPI1	b	b	b	b	b	b	b	b	b	a	a	a	a	a

[a] All isolates were monomorphic for an additional eleven enzymes: CAT1, DIA1, FUM1, G6PD1, GDH1, LAP1, MDH1, MDH2, MNR1, MNR2 and PEP1.

[b] For each electrophoretic type, the number of isolates and their hosts are shown in Table 4.

[c] Electromorphs were designated alphabetically in order of decreasing anodal migration.

[d] Electromorph 'n' of β-GLU1 is a non-resolving, low activity electromorph.

each of the other enzymes, the minimal staining was attributed to a unique electromorph with low activity, giving a total of six electromorphs for β-GLU1.

The enzyme AAT1, reported by Otrosina and Cobb (73) as having two electromorphs, had three electromorphs that were only revealed when stained slices of two different gel types were compared. On B2 gels, electromorph 'b' of AAT1 migrated at the same rate as electromorph 'a'. On D gels, however, electromorph 'b' migrated slower than electromorph 'a' but at the same rate as electromorph 'c'.

Four additional enzymes resolved after electrophoresis and staining but could not be used in the study. Activity of uridine diphosphoglucose pyrophosphorylase (EC 2.7.7.9) was minimal and could not be detected in many isolates used in the study. These incomplete data suggest that the enzyme is monomorphic in *L. wageneri*, as previously reported by Otrosina and Cobb (73). The banding pattern of acid phosphatase (EC 3.1.3.2) was also faint and apparently monomorphic. Conversely, the enzymes glutathione reductase (EC 1.6.4.2) and alcohol dehydrogenase (EC 1.1.1.1) stained well for most isolates but could not be used in this study due to extreme electrophoretic variability, with variation in the number of bands from isolate to isolate.

Successful crosses were not obtained in any of the pairings among *L. wageneri* isolates, so the actual genetic basis of electrophoretic variation in enzymes of *L. wageneri* could not be determined. In order to calculate Nei genetic distance and gene diversity, it was assumed that each of the 21 enzymes listed in Table 5 could be coded for by a different genetic locus, with different electromorphs representing the products of different alleles. This interpretation of the data

conforms to results from studies of isozyme variation in unrelated fungi where the genetic basis has been determined (14,34,61,88,90,94) and my own unpublished studies of isozyme variation in the related fungus *Ophiostoma nigrocarpum* (Davids.) deHoog.

Fourteen combinations of electromorphs (electrophoretic types) were detected among the 76 isolates tested (Table 6). Each type was found in only one variety of *L. wageneri*. Principal coordinate analysis of Nei genetic distances among the 14 electrophoretic types yielded three principal coordinates that contained 96.8 percent of the information of the original 14-variable distance matrix; the first, second, and third principal coordinates contained 53.5, 37.5, and 5.8% of the information in the matrix, respectively. The three principal coordinates were used to construct a three dimensional scatter plot to depict electrophoretic distance relationships among strains of *L. wageneri*. The scatter plot (Fig. 6) revealed three distinct clusters of electrophoretic types within the species. The clusters did not overlap and no intermediate types were found. The three clusters corresponded to the taxonomic varieties of Harrington and Cobb (44,45).

As shown in Fig. 4, each variety appeared to consist of one common electrophoretic type that was broadly distributed geographically and one or more types that were either geographically isolated from the common type or occurred in a local area within its range. Of the eight types found in var. *pseudotsugae*, H represented 74 percent of the isolates and had the broadest geographic occurrence. Four types (C, D, G, and I) were of local occurrence and three (E, F, and J) occurred at the edges of the range of the variety. Variety *ponderosum* had four electrophoretic types, with types K and L geographically distant from

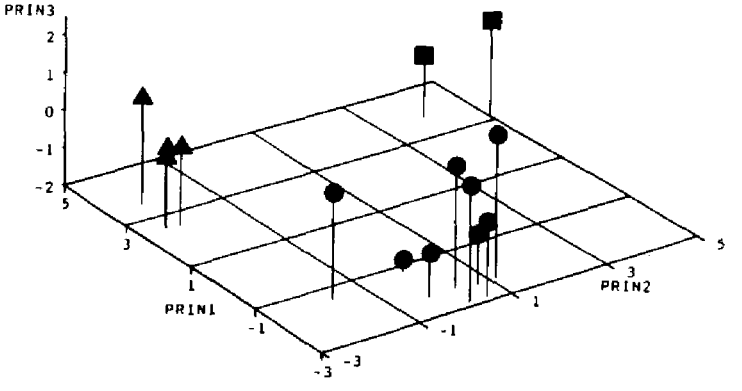

Figure 6. Ordination in three dimensions of electrophoretic distance relationships among electrophoretic types of the varieties of *L. wageneri*. Axes are the first three principal coordinates of the distance matrix transformed by principal coordinate analysis. Electrophoretic types of var. *wageneri* are represented by squares; var. *pseudotsugae* by circles; and var. *ponderosum* by triangles.

types M and N. In var. *wageneri*, electrophoretic type A was only found on a single plateau and differed from the more common type B only at the electromorph for PGM1.

The correspondence or lack of correspondence between electrophoretic distances among the electrophoretic types and their geographic distributions can be seen by comparing Fig. 4 with Figs. 6 and 7. Figure 4 shows considerable overlap between the distributions of the electrophoretic types of var. *pseudotsugae* and those of the other two varieties. Despite this geographic overlap, there are much greater differences among the electrophoretic types of different varieties than within varieties (Fig. 6). Within varieties, however, there is some correspondence between electrophoretic distances and geographic distributions of different electrophoretic types. The first three principal coordinates from principal coordinate analysis of subset distance matrices for var. *pseudotsugae* and var. *ponderosum* contained 94.2 and 100 percent of the information of the respective distance matrices and were used to construct the three dimensional scatter plots shown in Fig. 7. The first three principal coordinates contained 48.8, 28.9, and 16.5% of the variation in the distance matrix for var. *pseudotsugae*, and 71.8, 25.0, and 3.2% of the variation in the distance matrix for var. *ponderosum*.

In var. *pseudotsugae*, geographic separation (Fig. 4, middle) and electrophoretic distance (Fig. 7, top) were both small between types I and G, and large between type J and most other types. In other cases the electrophoretic distances were greater (e.g., between E and F) or smaller (e.g., between C and E) than would be expected on the basis of geographic separation alone. In var. *ponderosum*, there were only four

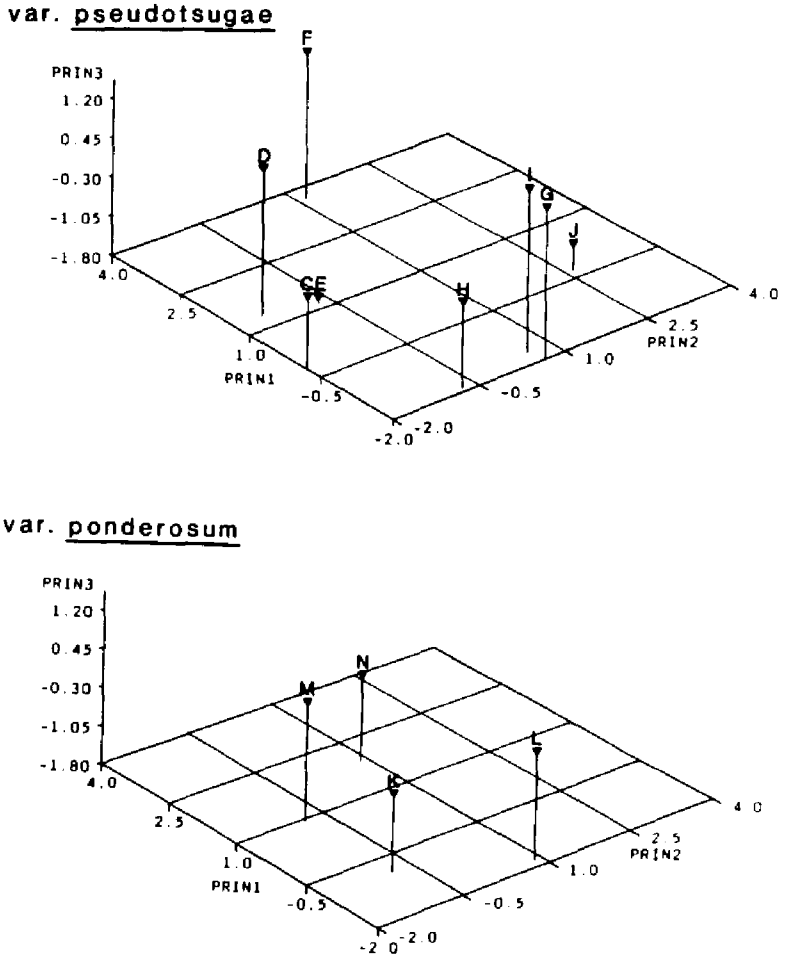

Figure 7. Ordination in three dimensions of electrophoretic distance relationships within varieties of *L. wageneri*. Top = var. *pseudotsugae*, bottom = var. *ponderosum*.

electrophoretic types and the variety was more restricted in geographic distribution (Fig. 4, top). There were also fewer isozyme differences (Table 6) and smaller electrophoretic distances (Fig. 7, bottom) among the types of var. *ponderosum*.

Nei gene diversity was low within each of the three varieties, ranging from 0.017 to 0.040 (Fig. 8). In contrast, there was a high degree of differentiation among varieties as indicated by large Nei distances among varieties and high coefficients of differentiation (R_{ST} and G_{ST}) for the species. The smallest Nei genetic distance calculated among varieties of *L. wageneri* was between var. *ponderosum* and var. *pseudotsugae*; the value of D = 0.257 estimates that an average of nearly 26 electrophoretically detectable substitutions would be found per hundred loci. The estimate of Nei interpopulational (intervarietal) gene diversity (R_{ST}) was 9.38, indicating that the amount of diversity occurring strictly among varieties was nine- to ten-fold greater than that occurring within varieties. Similarly, the Nei coefficient of gene differentiation (G_{ST}) was 0.860, indicating that 86 percent of the total electrophoretic diversity in the species was due to differences among varieties.

Discussion

Two types of analysis of the electrophoretic data (i.e., analysis of electrophoretic types separately and analysis of combined data from the types of each variety) strongly supported the division of the species into three taxa. The ordination of electrophoretic distance relationships among the electrophoretic types eliminated bias in assigning isolates to taxa prior to analysis and confirmed the lack of significant intermediates between the varieties. Further analysis of

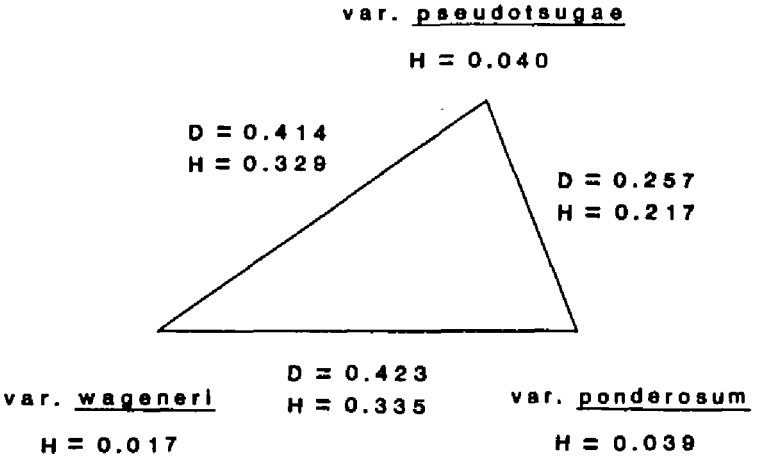

Figure 8. Relationships among varieties of *Leptographium wageneri* estimated from electrophoretic data. D = Nei (67) genetic distance between pairs of varieties. H = Nei (68) gene diversity calculated for varieties and pairs of varieties. H = 0.227 for the species.

pooled data at the varietal level gave estimates of Nei genetic distance, gene diversity, and gene differentiation based on an approximation of the relative frequency of the electrophoretic types as they occur in nature. The agreement of both methods of data analysis with the morphological and physiological differences reported by Harrington and Cobb (44,45) confirms the delineation of three distinct groups within *L. wageneri*.

These methods of isozyme analyses have also been of use in confirming the choice of the taxonomic rank for the variants. The choice of variety as the infraspecific rank was based on the occurrence of consistent but minor morphologic and physiological differences among isolates of the three variants, as well as their specialization to different hosts in nature. To some (48), the occurrence of minor but consistent differences is more characteristic of the rank of variety than of subspecies, in which morphologic differences are greater but intermediate forms may occur. The low number of polymorphic enzymes in the species and the lack of electrophoretic intermediates between the clusters in principal coordinate analysis might be considered consistent with this taxonomic concept of variety.

A review of isozyme studies of fish, amphibians, insects, mammals, and plants by Ayala (4) indicated that genetic distances between taxa of a particular taxonomic rank in one group of organisms may be higher or lower than comparisons of taxa in a different group of organisms. Using his tables, the Nei genetic distances observed between varieties of *L. wageneri* correspond to differences between subspecies in some groups of organisms and to sib-species or even distantly related species in other organisms. Furthermore, it is often difficult to

compare distances of different fungi because of differences in life cycle, ploidy, means of reproduction, environmental heterogeneity, and variability of the particular enzymes chosen for each study.

A comparison between this study and that of Otrosina and Cobb (73) showed agreement in the separation of *L. wageneri* into three varieties; in the smaller electrophoretic distance between var. *ponderosum* and var. *pseudotsugae* than between these two varieties and var. *wageneri*; and in identifying var. *pseudotsugae* as having the greatest, and var. *wageneri* as having the least amount of electromorphic variation. Significant areas of disagreement were the proportion of polymorphic enzymes found in the species as a whole (48% in this study, compared to 30% reported by Otrosina and Cobb), the aforementioned differences in interpretation of several enzymes, and the magnitude of electrophoretic distances between the varieties. Nei genetic distances calculated using Otrosina and Cobb's data were an average of 55% lower than values from this study. Many of these differences may be attributed to the higher number of isolates and enzymes utilized in the present study, which were required for a more detailed analysis. Nei (69) has suggested that accuracy in determining correct genetic distances and branching patterns in phylogenetic analysis is very low when the number of loci used is less than 20.

The presence or absence and/or frequency of occurrence of a teleomorph for *L. wageneri* has been a matter of debate since *O. wageneri* was first described from perithecia in bark beetle galleries in roots of ponderosa pine (36). Since that time, perithecia have not been reported to occur in cultures of *L. wageneri* or in black-stained material from nature, including material from the stand where the type

specimen was obtained (39,41). In this study, the teleomorph was not produced by pairing isolates of different electrophoretic types on media normally supporting the production of perithecia by *Ophiostoma* spp. Additional material is necessary to clarify the anamorph-teleomorph connection.

The question of whether *L. wageneri* commonly produces a teleomorph was also addressed by an examination of aspects of the isozyme data (i.e., the magnitude of electrophoretic distances between varieties and of diversity in the species and the pattern of distribution of electrophoretic types) that may be assumed to be affected by the presence or absence of recombination in the fungus. The magnitude of genetic distances between pairs of closely related species has been suggested to depend in part on the speed with which reproductive isolation mechanisms have developed and their efficacy during the process of speciation (4). If this is true, the large electrophoretic distances among varieties of *L. wageneri* may indicate the early initiation and effective operation of reproductive isolation. If one considers reproduction by strictly asexual means to be an extreme form of genetic isolation, the data may be interpreted as supporting the idea that the teleomorph is rare in nature.

The low number of electrophoretic types and the low diversity within varieties of *L. wageneri* also suggest a rarity or lack of sexual reproduction. Similarly, Roelfs and Groth (87) found that asexual populations of *Puccinia graminis* Pers. f.sp. *tritici* Eriks. & Henn. have far fewer virulence phenotypes (combinations of virulence traits) than do sexual populations, and the distribution of such phenotypes was extremely non-random. Genetic diversity has also been reported to be

lower in asexual populations of *Phytophthora infestans* (Mont.) de Bary (99) and *Magnaportha grisea* (Hebert) Barr (57) than in populations of the same species in which sexual reproduction occurs.

Finally, the widespread occurrence of some of the electrophoretic types of *L. wageneri* can also be interpreted as evidence for the lack of sexual recombination in this fungus. Although selection acts on the level of the allele or allelic combination in an organism with sexual recombination, the genome may be the unit of selection in strictly asexual organisms, resulting in competition among clones (25). Widespread occurrence of a very few electrophoretic types and geographic isolation of others, as was found in the varieties of *L. wageneri*, has also been found in the asexual pathogen *Alternaria mali* Roberts (50) and in asexual populations of *Magnaportha grisea* (57).

Maynard Smith (60) has suggested that the development of a strictly asexual mode of reproduction may not be detrimental to a population or species if progeny are 1) consistantly dispersed into an environment similar to that of the parent, 2) if there is intense selection pressure, and 3) if populations are very small. An examination of the life cycle of *L. wageneri* reveals that this species may meet all three of these criteria.

L. wageneri occurs in nature in discrete infection centers. Initial infections are established as root feeding bark beetles transport the fungus to new areas (17,46,108). The pathogen/vector relationship is not strong, however, making the establishment of a new infection center a rare event, especially establishment of infection centers in increasingly distant areas. Spread within an infection center is primarily by limited growth of mycelium through soil between

infected and uninfected roots, or more rarely, by direct root contact (17). Aside from brief periods of movement between roots or as spores carried on the exoskeletons of insect vectors, *L. wageneri* exists only in the xylem of living or recently killed trees. Thus, the hosts of *L. wageneri* may provide a relatively uniform and protected environment for the fungus from generation to generation, meeting Maynard Smith's first criterion. Secondly, the host specialization of the three varieties suggests that host-pathogen interactions exert strong selective pressure on the fungus. Since genetic differences are immediately expressed in organisms with a predominant haploid stage in the life cycle (e.g., Deuteromycotina and most Ascomycotina), selection pressure could be expected to be particularly intense. Thirdly, the dependence of the fungus on an uncertain vector provides that population size is at least intermittantly low. Although *L. wageneri* infection centers covering several hectares may develop through root-to-root spread without the aid of vectors (18), the founding of each new infection center could be seen as a genetic bottleneck and a great reduction in effective population size.

In spite of the limited variation found in *L. wageneri*, it is possible that the fungus is a sexually competent, heterothallic species with an uneven distribution of mating types. Because of the low probability of establishing infection centers, there may be a high probability of losing one mating type during establishment of any infection center. Mating types could become geographically isolated in this way, with rare sexual recombination limited to regions of contact between infection centers carrying the complementary mating types. The rarity of one mating type has been noted in epidemic populations

of the related fungus *Ophiostoma ulmi* (Buisman) Nannf. (13) and only one mating type is found in populations of *Phytophthora infestans* (Mont.) de Bary in Europe and North America (33). These asexual populations of *P. infestans* have lower electrophoretic diversity than do populations from central Mexico in which both mating types are found (99).

The lack of identifiable regions of high variability in *L. wageneri* and the lack of ability of paired isolates from different locations and/or different electrophoretic types to form perithecia in culture argue against the possibility of a rare mating type in this fungus. If the suggested scenario of predominantly asexual reproduction is accurate, it would be more likely that an organism would lose the ability to express loci for unused sexual capabilities over time through either random fixation of mutations or through selection of asexual strains without the "cost of sex" (60) associated with maintaining active alleles at those loci.

In conclusion, the results of isozyme study in *Leptographium wageneri* have supported the division of the species into three taxonomic varieties and suggest that sexual recombination is rare or lacking in this fungus.

CHAPTER III

HETEROKARYOSIS AND VEGETATIVE COMPATIBILITY IN *LEPTOGRAPHIUM WAGENERI*

Introduction

Leptographium wageneri (Kendrick) Wingfield is the cause of a unique vascular wilt of conifers, black stain root disease (17). The disease causes tree mortality in western North America and occurs in discrete infection centers by growth of the fungus for short distances through the soil between roots of adjacent trees (35,49). Infection centers are established when arthropod vectors, primarily root-feeding bark beetles in the genus *Hylastes* (Coleoptera: Scolytidae), carry sticky masses of spores from conidiophores within beetle galleries of colonized trees to uninfected trees.

Three host-specialized varieties of *Leptographium wageneri* are recognized, based on differences in conidiophore morphology, cultural appearance, and temperature maxima for growth (44,45). *Leptographium wageneri* var. *wageneri* infects pinyons (*Pinus edulis* Engelm. and *P. monophylla* Torr. & Frem.); var. *ponderosum* (Harrington & Cobb) Harrington & Cobb is pathogenic to hard pines (*P. contorta* Dougl., *P. ponderosa* Laws., and *P. jeffreyi* Grev. & Balf.); and var. *pseudotsugae* Harrington & Cobb is a pathogen of Douglas-fir (*Pseudotsugae menziesii* (Mirb.) Franco).

In recent studies of genetic variation in *L. wageneri* using enzyme electrophoresis, Otrosina and Cobb (73) and Zambino (Chapter 2) found that differences among varieties accounted for most of the limited

genetic diversity in the species. Gene diversity (Nei's H) was 0.227 for the species, and only 0.017, 0.039, and 0.040 for var. *wageneri*, var. *ponderosum*, and var. *pseudotsugae*, respectively (Chapter 2). Also, only 14 combinations of putative alleles from 21 enzymes were detected among 76 strains of this fungus. Each of these electromorph combinations or "electrophoretic types" was restricted to a single variety.

Testing for vegetative compatibility, i.e., the ability of hyphae of different strains to anastamose and produce cells containing nuclei of both strains, is another method that has been used to study population structure in a number of imperfect and ascomycete fungi (3,10,12,13,21,32,51,52,66,76,79,82). In one method, vegetative compatibility is indicated by the development of an interaction zone with vigorous mycelial growth due to complementation between auxotrophic mutants derived from different fungus strains paired on minimal medium. The selection of auxotrophic mutants deficient in permeases or metabolic enzymes has been facilitated by the use of toxic substrate analogues (20,22,53,92).

Working with *Emericella nidulans* (Eidam) Vuillemin (anamorph = *Aspergillus nidulans* (Eidam) Winter), Cove (22) identified fast-growing sectors that developed on media containing the nitrate analogue chlorate to be a useful source of complementary mutants unable to use nitrate as a nitrogen source. The metabolized products of chlorate are toxic, so only sectors deficient for chlorate metabolism and auxotrophic for nitrate metabolism are able to grow on chlorate media. Cove (22.23) recovered three complementary classes of mutants having mutations at the locus for nitrate reductase (E.C. 1.6.6.2), at a locus

that regulates the production of the enzymes nitrate reductase and nitrite reductase (E.C. 1.6.6.4) of the nitrate metabolism pathway, and at any of five different loci associated with the production of the cofactor for nitrate reductase. Since the cofactor is also required for activity of xanthine dehydrogenase (E.C. 1.1.1.204), the latter mutants also have decreased ability to metabolize xanthine and hypoxanthine as nitrogen sources.

Puhalla (81) demonstrated the usefulness of such mutants in a study of vegetative compatibility among strains of different races and formae speciales of *Fusarium oxysporum* Schlecht. Studies of vegetative compatibility using nitrate non-utilizing mutants have since been made in species and subspecies of additional ascomycetes and fungi imperfecti (12,20,21,32,51,53,76).

This study examined vegetative compatibility between strains of *L. wageneri* based on complementation of nitrate non-utilizing strains. The occurrence of vegetative compatibility groups (groups of strains that anastamose and can complement one another) was compared with other phenotypic differences (electrophoretic types) in order to better understand the inherent population structure of this fungus.

Materials and Methods.

Strains used.

Seventy-six strains were selected to broadly represent the hosts and geographic areas where the fungus has been reported (Table 7). Each strain originated from a different infection center.

Media.

The following media were adapted from the media of Cove (22), Puhalla (81), and Correll *et al.* (20). Basal medium (BM) contained

TABLE 7. VEGETATIVE COMPATIBILITY GROUPS AND ELECTROPHORETIC TYPES OF WILD-TYPE STRAINS OF *LEPTOGRAPHIUM WAGENERI*.

VC Group	Electrophoretic Type[a]	Strains[b]
var. *wageneri*		
I	A	CAS4 (ATCC 64194), CAS5, CAS7, CAS9
	B	CAS1 (ATCC 64193), CAS2, CAS3, CAS15 (ATCC 64195), COE1 (ATCC 58576) COE2, COE6, COEN, IDE1, NES1 (ATCC 64192), NES2, NES3, NES4, NME1 (ATCC 58579), UTE1
var. *pseudotsugae*		
II	H	BCD1 (ATCC 58574)
III	H	BCD11
IVa	H	BCDJ, CAD30
	G	BCH1 (ATCC 42953)
IVb	H	CAD32, ORD5
	I	ORDQ, WADU
IVc	H	CAD19, ORD4, ORDP
V	H	ORD1
VI	H	ORD2
VII	C	IDD2
VIIIa	H	CAD40, CAD56
VIIIb	H	CAD1, CAD2, CAD6, CAD18 (ATCC 64196), CAD27, CADF
IX	H	CAD22, CAD31
X	H	CAD5, CAD55
XI	H	CADX
NC[c]	H	IDD1, ORD3
	C	MOD22
	D	MOD1 (ATCC 58578)
	E	NMD1
	F	NMD2
	J	COD2 (ATCC 64191)

TABLE 7, continued.

var. *ponderosum*		
XII	K	BCL1 (ATCC 42954), BCL2, BCW1, BCL4, MOW2
	L	BCL3
XIII	M	CAJ3, CAP3, CAP19 (ATCC 58575), CAP36, CAPC, CAPD, CAPH, CAPI, CAPW
	N	CAPY, ORL1, ORP1
XIV	M	ORMS (ATCC 58581)
NC[c]	M	IDP1 (ATCC 58577)
	N	ORH1 (ATCC 58580)
NT[d]	M	CAJ1

[a] From Table 4 of Chapter 2.

[b] Culture numbers are those used in the collection of T. C. Harrington. The first two letters designate the state or province of origin. Numbers in parentheses are those of the American Type Culture Collection.

[c] Non-complementing strains; only one phenotype recovered.

[d] Not tested due to unusual growth characteristics.

20.0 g glucose, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCL, 0.1 g $CaCl_2$, 0.2 ml of the trace element solution of Correll *et al.* (20), 10.0 ml of a vitamin solution (0.1 mg thiamine HCl, 0.075 mg pyridoxine HCl, and 0.005 mg biotin in 1.0 ml of 50% ethanol), and 20.0 g agar-agar (Sigma Chemical Co.) per liter of distilled H_2O. Complete medium (CM), hypoxanthine medium (HM), and nitrate minimal medium (MM) were prepared by adding 1.0 g l-asparagine, 0.2 g hypoxanthine, or 1.0 g $NaNO_3$, respectively, to 1.0 l of basal medium. Nitrite medium (NM) was prepared by adding 0.1 g $NaNO_2$ to 1.0 l of basal medium and substituting 25 g agar noble (Difco Laboratories) for the 20 g of agar-agar. The media CMT, HMT, MMT, and NMT were CM, HM, MM, and NM to which 2 ml of 10% Triton X-100 was added to reduce radial growth rate. Weak nitrate medium contained 30.0 g agar noble (Difco Laboratories), 5.0 g glucose, 0.5 g $NaNO_3$, and the vitamins and trace elements as used in MM and other media.

Nuclear staining.

The number of nuclei in hyphae, hyphal tips, conidia, and developing conidiophores was determined in several strains of *L. wageneri* var. *pseudotsugae*. Strains were grown at 19°C on weak nitrate medium and MMT, on sterile cellophane strips placed on the two media, and on sterile slides that had been coated with these media. Slides and cellophane strips that contained young hyphae and pieces of cultures that contained young conidiophores were stained with HCl-Giemsa (5) or with Safranin O-KOH (6). Most microscopic observations of hyphae were of cellophane strip cultures stained with Giemsa. Nuclei of conidia and immature conidiophores were best observed in materials stained with Safranin O-KOH.

Mutant selection and characterization.

Inocula of wild-type strains were grown at 19°C on either 1.5% malt extract agar (MEA) that contained 0.05% yeast extract (MYEA) or on CM. Mycelial plugs, 9 mm in diameter, were then transferred to MYEA and CM that contained 1.5 percent $KClO_4$ and incubated at 19°C. Fast-growing sectors that developed after 3 to 8 weeks were subcultured to fresh plates of MYEA containing chlorate. Each mutant strain was labelled with the wild-type designation plus a suffix indicating chlorate resistance (C1, C2, etc.), and a hyphal-tip culture of each mutant was stored on a MEA slant.

Mutants recovered early in the study were phenotyped for nitrogen-source utilization by observing three weeks of growth on BM, CM, MM, NM, and HM, but it was found that media containing Triton X-100 were more effective in differentiating phenotypes. Criteria for reduced utilization of a nitrogen source were slower and/or less dense growth in mutant strains than in parental wild-type strains and/or the lack of a difference in a mutant's growth on a nitrogen-source medium versus basal medium that contained no nitrogen source.

Mutants were classified by phenotype as discussed by Cove (23) and Correll *et al.* (20). Three mutant classes were designated following suggested genetic nomenclature (110) and current usage in other plant pathogenic fungi (20,32,53): nit1 mutants had sub-optimal growth only on nitrate media and were presumed to have a mutation at the gene for nitrate reductase. Nit3 mutants had sub-optimal growth on both nitrate and nitrite media and were presumed to have a mutation in the gene regulating the production of nitrate reductase and nitrite reductase. NitM mutants had sub-optimal growth on hypoxanthine and nitrate media

and were presumed to be deficient for the enzyme cofactor of nitrate reductase.

Complementation Tests.

After 14 days growth on CM, 2.0 x 2.0 mm mycelial plugs of a pair of mutant strains were placed adjacent to each other in the center of a 60 mm plate of MMT. Plates were examined after 6, 9, and 12 weeks incubation at 19°C for the presence of a broad band of dense, aerial mycelium along the line of confrontation that would indicate anastamosis and complementation. Self-pairings of mutants and self-pairings of wild-type strains were used as references for auxotrophic versus prototrophic growth.

In many pairings, including all pairings with poorly defined zones of complementation, 1.5 x 1.5 mm plugs of colonized agar were transferred after 6 or more weeks from the line of contact between the mutants and from either side of the paired inoculum blocks, where mycelia of the mutants had not intermingled. Colonies from the transfers were examined after 3 weeks incubation at 19°C on MMT for differences in growth.

Tests for heterokaryosis.

Attempts were made to recover heterokaryons (i.e., cells with nuclei of more than one genotype) from several pairings that showed good complementation. Transfers of single hyphal tips from prototrophic subcultures were made from pairings of each of the three varieties; in var. *pseudotsugae*, transfers were also made from masses of conidia from individual conidiophores. To obtain hyphal tips, plugs of mycelium from the growing edge of complementing colonies were transferred to weak nitrate medium. After 5 to 9 days at 19°C, plates

were examined at 40x under a dissecting microscope and individual hyphal tips were excised from the edge of the colony and transferred to MMT. Cultures that grew from these hyphal tips were "primary hyphal-tip cultures". Each primary hyphal-tip culture that showed prototrophic growth on MMT was used to obtain additional hyphal-tip cultures (secondary hyphal-tip cultures). Nitrogen utilization phenotypes were determined for all cultures derived from hyphal tips or conidia after 3 weeks growth on MMT and HMT.

Using starch gel electrophoresis (buffer system A, Chapter 2), the electromorphs of the enzymes β-glucosidase (E.C. 3.2.1.21) and esterase (E.C. 3.1.1.1) were determined for hyphal-tip cultures of some pairings. In these pairings, electromorphs were determined for all primary hyphal-tip cultures that were prototrophic on MMT and for some of the auxotrophic and prototrophic, secondary hyphal-tip cultures. Enzymes were extracted from cultures grown for 16 days in 30 ml of MM lacking agar or liquid MM medium amended with 0.05 g/l asparagine to allow adequate growth of nitrate non-utilizing strains.

Macronematous conidiophores were produced on 2.0 x 2.0 mm plugs of colonized MMT transferred onto weak nitrate medium. After 8 to 10 days incubation, plates were examined at 40x under the dissecting microscope. A sterile needle was used to transfer masses of conidia from individual conidiophores to plates of MMT. Phenotypes were determined for 2-6 sectors and/or other selected locations at the edge of each colony.

Cultures derived from single conidia were obtained by streaking a mass of conidia from an individual conidiophore onto a plate of MEA.

After two days of incubation at 19°C, germinating conidia were individually transferred to MMT and later phenotyped on MMT and HMT.

Results

Nuclear condition and anastamosis. Microscopic examination of six strains of var. *pseudotsugae* indicated that cells of the hyphae and hyphal tip cells are multinucleate (Fig. 9a, 9b). For example, in strain WADU, hyphal tip cells from three hyphae had an average of 10 nuclei, and the average number of nuclei in the first seven cells of one hypha was 11 nuclei per cell, with a range of 4 to 17 nuclei per cell. This multinucleate condition was also observed in unbranched initials (stipes) of macronematous conidiophores (Fig. 9c).

Young conidia, (i.e., terminal conidia still attached to the conidiogenous cells of mature conidiophores) were invariably uninucleate, but the binucleate condition was occasionally seen in older conidia that were either still attached to the conidiophore or that had begun to germinate in the droplet of spores adhering to the conidiophore (Fig. 9d).

Anastamoses appeared to be common, but were most frequent whenever hyphae grew in a parallel orientation (Fig. 9b). Anastamoses also appeared to be more frequent in older, more heavily colonized parts of the culture and were rare in hyphal tips at the edge of the culture.

Mutant recovery and characterization of mutants.

Nitrate non-utilizing mutants were obtained from all of the 76 selected strains of *L. wageneri*. NitM mutants (cofactor mutants) of var. *pseudotsugae* and var. *ponderosum* were readily distinguished from other nitrate non-utilizing mutants by their slower growth on HMT, and in var. *pseudotsugae*, by the production of fewer conidiophores.

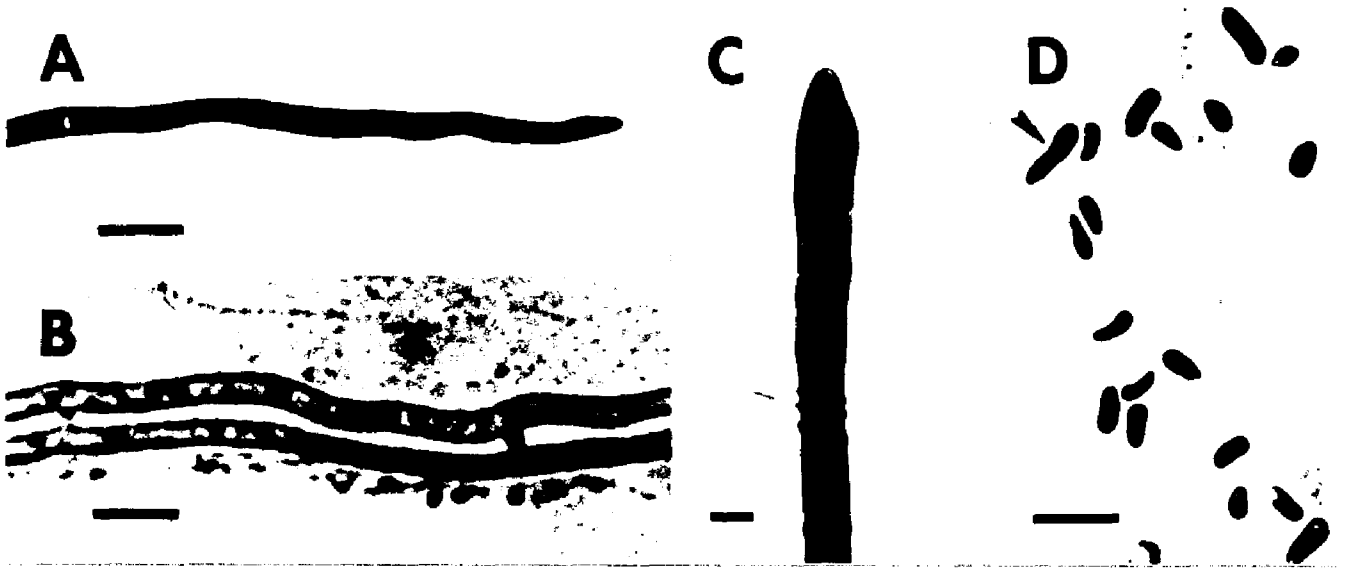

Figure 9. Stained nuclei of *L. wageneri* var. *pseudotsugae*. A. Multinucleate hyphal tip of strain WADU stained with Safranin O. B. Multinucleate, anastamosing hyphae of strain CAD30 stained with Giemsa. C. Growing tip of a multinucleate, developing conidiophore of ORD5 stained with Safranin O. D. Uninucleate conidia and a germinating, binucleate conidium (arrow) from the mutant pairing CAD32C1xORDQC1 stained with Safranin O. The bar in each photograph represents 10 micrometers.

However, it was difficult to detect nitM mutants of var. *wageneri*, regardless of whether Triton X-100 was used in the phenotype media; these mutants had only slightly less radial growth than wild-type strains on HMT.

Although different concentrations of nitrite were tested, sub-optimal utilization of nitrite was difficult to detect, apparently because of the toxicity of nitrite. In var. *pseudotsugae*, nit3 mutants (mutants deficient at the regulatory locus for nitrate reductase and nitrite reductase) had very thin growth on NM and NMT, and wild types could only be distinguished by their slightly denser growth. In var. *ponderosum*, however, the difference was less apparent. In var. *wageneri*, growth of all wild-type and mutant strains was poor on NM and NMT, with less expansive growth than occurred on the basal medium that lacked nitrogen.

NitM mutants were the most commonly recovered mutants in var. *wageneri*, with five or more complementary types of nitM mutants indicated from the results of pairings in this variety (Table 8). Nit1 mutants (nitrate reductase mutants) were the most frequently recovered mutants in var. *ponderosum* and var. *pseudotsugae*. NitM mutants were rare in var. *ponderosum*.

Complementation and heterokaryosis.

On plates of MMT, a zone of dense hyphal growth, indicating complementation, occurred in the region of contact between mutants in some pairings (Fig. 10). Complementation was more rapid and the zone of complementation more distinct on this medium than in preliminary pairings on other media (MM or MM plus 0.25 or 0.5 % sorbose). Growth inhibition caused by the Triton X-100 may have increased opportunity

TABLE 8. COMPLEMENTATION AMONG nitM MUTANTS OF LEPTOGRAPHIUM WAGENERI VAR. WAGENERI.

Mutant	Phenotype[a]	CAS1C1 nitM1	CAS3C2 nitM5	CAS4C7 nitM5	CAS7C6 nitM2	CAS9C1 nitM3	CAS15C4 nitM2	COE1C1 nitM2	COE2C6 nitM2	IDE1C3 nitM2	NES2C1 nitM2	NES3C3 nitM	NME1C3 nitM2	UTE1C2 nitM5
CAS1C1	nitM1	-[b]	+	+	+	+	+	+	+	+	ND	-	+	+
CAS1C2	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	+
CAS2C5	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	+
CAS3C1	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	+
CAS4C2	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	+
CAS5C1	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	S+
CAS5C4	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	+
CAS7C1	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	+
CAS9C1	nitM3	+	+	+	+	-	+	+	+	+	+	-	+	+
CAS9C8	nitM4	+	+	+	+	+	+	+	+	+	+	-	+	+
CAS15C2	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	+
CAS15C3	nitM2	+	+	+	S+	+	-	-	-	-	-	-	-	+
COE1C3	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	+
COE6C2	nitM2	+	+	+	S+	+	-	-	-	-	-	-	-	+
COENC1	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	+
IDE1C1	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	+
NES1C1	nitM2	+	+	+	S+	+	-	-	-	-	-	-	-	+
NES2C1	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	+
NES3C1	nitM	-	-	-	-	S+	-	-	-	-	-	-	-	-
NES4C3	nitM2	+	+	+	S+	+	-	-	-	-	-	-	-	+
NME1C2	nitM2	+	+	+	S+	+	-	-	-	-	-	-	-	+
UTE1C7	nitM2	+	+	+	-	+	-	-	-	-	-	-	-	+

[a] NitM phenotypes based on growth on media containing nitrate, nitrite, and hypoxanthine. The numeric suffix 1-5 was used to designate different types of nitM mutants, as determined by differences in complementation.

[b] Complementation (+) was determined by the development of a dense zone of hyphal growth on MMT after 6 weeks. Pairings with "slow complementation" (S+) developed a faint zone of complementation after 6 weeks or a dense zone after 9 to 12 weeks. Pairings with slow, appressed growth after 12 weeks were labelled as negative (-). Complementation was not determined in pairings marked ND.

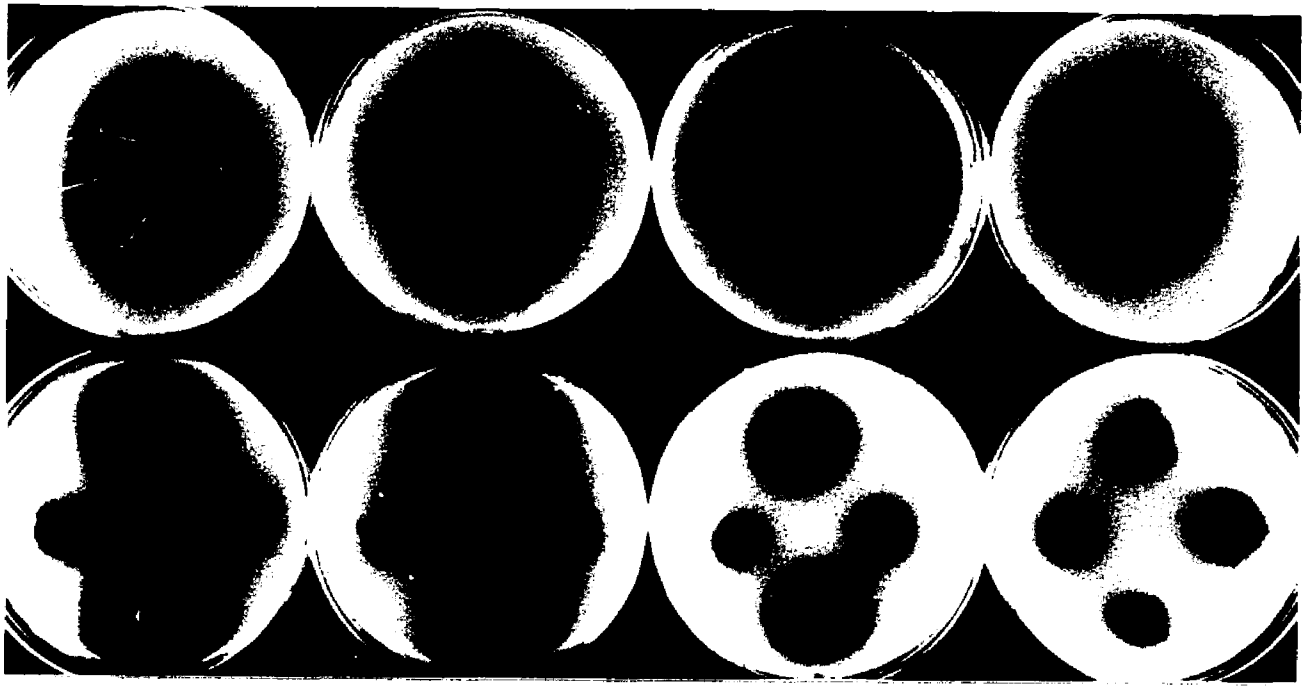

Figure 10. Pairings between nitrate non-utilizing mutants of *L. wageneri* var. *ponderosum* (top), and mycelial transfers from the respective pairings (bottom) on minimal medium containing nitrate and Triton X-100. Arrows in upper left designate areas from which subcultures were taken. From left to right are pairings CAP19C2xCAP36C1 and BCW1C3xMOW2C2, both positive (+) for complementation; "slow positive" (S+) pairing CAPIC5xORL1C5; and negative (-) pairing BCL1C1xCAPDC1.

for contact between hyphae. Pairings that had dialysis membrane separating the hyphae of complementing mutants did not develop the zone of dense hyphal growth, except where hyphae of both mutants had grown past the edge of this semi-permeable barrier and made direct contact.

Pairings were considered positive (+) for anastamosis and complementation if a dense zone of surface and aerial hyphae developed in the area of contact between the mutants after 6 weeks (Fig. 10). In positive pairings, colonies from transfers from the zone of contact were denser, fluffier, and had a faster rate of radial growth after 3 weeks incubation on MMT than did colonies transferred from the unmingled mutant mycelia. A pairing was labelled a "slow positive" (S+) if the zone of complementation was thin or spotty at 6 weeks and/or if complementation was only evident after 9 or more weeks and there was significantly better growth in colonies transferred from the zone of contact than in transfers from the mutants. If a barely distinguishable zone of complementation developed after 9 or more weeks and there were no differences among the transfers, the pairing was labelled as a "slow negative" (S-). Pairings that lacked visible complementation at 12 weeks were labelled as negative (-).

Most (88 and 58%, respectively) of the hyphal tips obtained from complementing pairings of var. *ponderosum* and var. *wageneri* failed to grow. Of the 59 primary hyphal-tip cultures obtained from pairings of var. *wageneri* (CAS1C1x CAS5C2 and CAS3C1xCAS4C7) and the 16 primary hyphal-tip cultures obtained from pairings of var. *ponderosum* (BCL1C1x BCL1C2 and BCL3C11xBCW1C3), none were prototrophic.

Most of the transferred hyphal tips of var. *pseudotsugae* survived; 69% of the original hyphal-tip transfers from five pairings grew to

form primary hyphal-tip cultures. The percentage of prototrophs was low in both the primary hyphal-tip cultures (0 to 19%) and in the derived, secondary hyphal-tip cultures (4 to 11%) of the five tested pairings of var. *pseudotsugae* (Table 9). Subcultures from the prototrophic, primary hyphal-tip cultures had various degrees of sectoring after three weeks growth on MMT (Fig. 11); some subcultures appeared to be totally auxotrophic.

Each of 13 prototrophic, primary hyphal-tip cultures of var. *pseudotsugae* (Table 9) had double banding patterns for β-glucosidase and/or esterase, with the two bands co-migrating with electromorphs of the two wild type strains represented in the pairing (Fig. 12).

For each of the selected pairings, later subcultures from the two primary hyphal-tip cultures that had the least sectoring and 29-32 secondary hyphal-tip cultures derived from the two subcultures were also characterized electrophoretically. Twenty-two cultures (13 subcultures and 9 secondary hyphal-tip cultures) appeared to be prototrophic on MMT; the rest were auxotrophic. All strains were grown on liquid MM medium containing some asparagine, a medium less selective for prototrophic growth. The 102 auxotrophic secondary hyphal tip cultures invariably had electrophoretic patterns that matched those of the parent mutant with the same nit phenotype (no non-parental types were recovered). In contrast to the double-banding isozyme pattern seen in each of the 13 hyphal tip cultures grown in liquid MM medium lacking asparagine, only 8 of the 22 prototrophic cultures had similar double-banded patterns when grown on liquid medium containing asparagine. The other 14 prototrophs either had one band that was much fainter than the other (7 cultures), or one band of the pair was

TABLE 9. NUMBER OF CULTURES HAVING MUTANT (AUXOTROPHIC) VERSUS WILD-TYPE (PROTOTROPHIC) PHENOTYPES[a] OBTAINED FROM SELECTED PAIRINGS BETWEEN nitM AND nit1 MUTANTS OF LEPTOGRAPHIUM WAGENERI VAR. PSEUDOTSUGAE.

Pairings	Primary Hyphal-Tip Cultures[b]			Secondary Hyphal-Tip Cultures[b]			Cultures from Single Conidiophores[c]		
	Proto-trophs	nitM mutants	nit1 mutants	Proto-trophs	nitM mutants	nit1 mutants	Proto-trophs	nitM mutants	nit1 mutants
CAD30C1:BCH1C3[d]	2	0	33	2	0	25	NT[e]	NT	NT
ORDQC1:CAD32C1	6	25	1	4	41	32	10	17	0
ORDQC1:ORD5C2	2	12	9	1	11	13	13	26	2
WADUC3:CAD32C1	0	37	0	NT	NT	NT	NT	NT	NT
WADUC3:ORD5C1	3	23	9	6	15	32	33	34	2

[a] Phenotypes determined on the media MMT and HMT.

[b] For primary hyphal-tip cultures, hyphal tips were from the edge of subcultures from a mutant pairing after 5-9 days growth on weak nitrate medium; secondary hyphal-tip cultures were similarly obtained from primary hyphal-tip cultures that had prototrophic (wild-type) growth on MMT medium.

[c] Established by transferring masses of conidia from single conidiophores that formed in subcultures from the mutant pairings after 8 to 10 days growth on weak nitrate medium.

[d] For each designated pairing, the phenotypes of the mutants preceding and following the colon are nitM and nit1, respectively.

[e] Not tested.

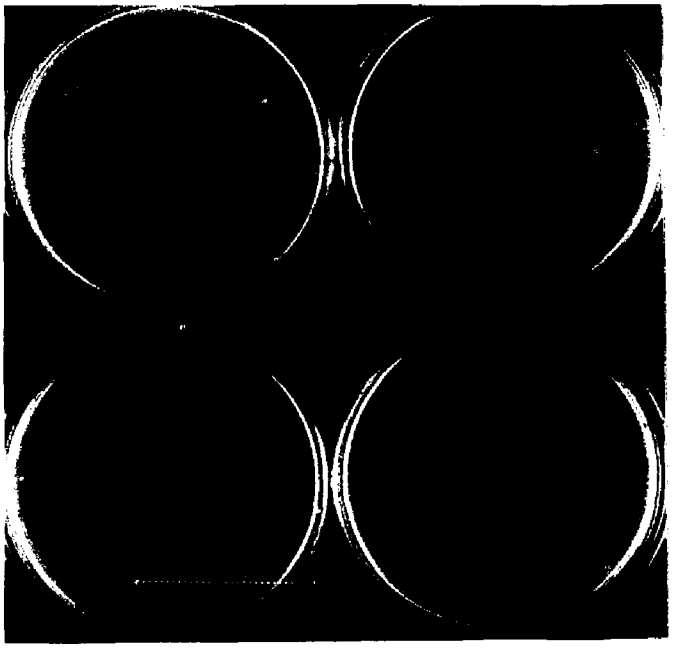

Figure 11. Subcultures from prototrophic hyphal-tip cultures derived from pairings CAD32C1xORDQCI (top left), ORD5C1xORDQC1 (top right), and ORD5C1xWADUC3 (bottom, left and right) of var. *pseudotsugae* grown on MMT. The subcultures at top and at bottom left have auxotrophic (flat) and prototrophic (fluffy) sectors; the subculture at bottom right is totally lacking in prototrophic growth.

Figure 12. Isozyme patterns of β-glucosidase for strains of *L. wageneri* var. *pseudotsugae*. Single bands at far left and far right are from wild-type strains CAD32 and ORDQ, respectively; double bands are from prototrophic cultures obtained from single hyphal tips of the mutant pairing CAD32C1xORDQC1.

completely undetected (7 cultures).

In 21 of the 22 originally prototrophic cultures grown on asparagine, isolations were made from chromatography-paper wicks of enzyme extracts that had been prepared for use as electrophoresis samples. The ultrafrozen wicks were plated onto MMT containing 200 ppm cycloheximide to supress growth of contaminants, and prototrophic and auxotrophic sectors that developed were transferred to MMT and HMT for phenotyping. Cultures derived from wicks of five of the prototrophic cultures yielded one or more prototrophic sectors and sectors of both auxotrophic phenotypes, 12 cultures yielded prototrophs and one of the auxotrophic phenotypes, and 4 yielded only one of the auxotrophic phenotypes. In most cases, the double banded electrophoretic patterns with the two bands of nearly equal intensity were from mycelial extracts of cultures that had the highest proportion of prototrophic sectors or had prototrophs and both auxotrophic phenotypes among the sectors when the wicks were plated on MMT.

Isolations from masses of conidia from single conidiophores developing on areas of complementation yielded significantly more prototrophic colonies than did isolations from hyphal tips (Table 9, $p < 0.005$ in Chi-square tests of contingency for each pairing). However, 287 single-spore colonies derived from six conidiophores were all auxotrophic, even though each of the six conidiophores produced conidia of both mutant types used in the pairings. The ratio of occurrence of the rarer vs. the more common auxotroph in these single-spore isolations from single conidiophores ranged from 1:55 to 18:27.

Vegetative Compatibility Groups.

Strains of the two electrophoretic types of var. *wageneri*, which

differ in isozymes of phosphoglucomutase (EC 5.4.2.2), appeared to be fully compatible, i.e., of a single VC group (Table 8). Most negative pairings could be attributed to the mutants of the pairing having mutations in the same nitM gene. However, pairings of mutants C1 and C3 of NES3 with other mutants and with themselves resulted in only one slow positive pairing, between CAS9C1 and NES3C1 (Table 8).

Three VC groups were apparent in var. *ponderosum* (Table 10) and these three groups had unique geographic distributions (Fig. 13). One VC group (VC group XII) contained all of the strains of the northern Rocky Mountains except a strain from Idaho; these strains were of two electrophoretic types that differed in isozymes of glucose-phosphate isomerase (EC 5.3.1.9). A second large VC group (VC group XIII) contained two additional electrophoretic types that differed in isozymes of β-glucosidase (Table 7). The third VC group contained a single self-compatible strain (ORMS). Nit mutants of two additional strains (IDP1 and ORH1) did not complement other strains of var. *ponderosum* and could not be tested for self-compatibility due to recovery of only one mutant phenotype. Another strain (CAJ1) and its mutants could not be used due to poor growth.

In an initial set of pairings of var. *pseudotsugae*, transfers were made from all pairings. For each nit1 or nitM mutant that did not complement any of the mutants in the initial pairing, an additional nit1 or nitM mutant previously generated from the wild type strain was used in a second set of pairings. In this second set, the new mutants were paired with all of the mutants used in the initial pairing and pairings between putative VC groups or subgroups that showed limited inter-group complementation (slow positives) in the first set of

TABLE 10. COMPLEMENTATION AMONG NITRATE NON-UTILIZING MUTANTS OF THREE VEGETATIVE COMPATIBILITY GROUPS (VC GROUPS) OF LEPTOGRAPHIUM WAGENERI VAR. PONDEROSUM.

Mutant	Phenotype[a]	VC group	BCL1C2 nit3 XII	BCL2C1 nit3 XII	BCL3C11 nitM XII	BCW1C3 nitM XII	CAP19C1 nit1 XIII	CAP36C1 nit3 XIII	CAPCC2 nit? XIII	CAPDC1 nit1 XIII	CAPIC5 nit? XIII	CAPWC3 nit3 XIII	ORLIC7 nit3 XIII	ORMSC2 nit3 XIV
BCL1C1	nit1	XII	+[b]	+	+	+	-	-	-	-	-	-	-	-
BCL1C2	nit3	XII	-	-	+	+	-	-	-	-	-	-	-	-
BCL3C5	nit?	XII	-	-	+	+	-	-	-	-	-	-	-	-
BCL3C11	nitM	XII	+	S+	-	+	-	-	-	-	-	-	-	-
BCL4C1	nit1	XII	S+	S+	+	+	-	-	-	-	-	-	-	-
BCW1C1	nit?	XII	-	-	+	S+	-	-	-	-	-	-	-	-
BCW1C3	nitM	XII	+	+	+	-	-	-	-	-	-	-	-	-
MOW2C2	nit?	XII	-	-	S+	S+	-	-	-	-	-	-	-	-
CAJ3C2	nit1	XIII	-	-	-	-	+	+	S+	S+	S+	+	+	-
CAP3C1	nit1	XIII	-	-	-	-	-	S+	-	-	-	-	-	-
CAP19C2	nit1	XIII	-	-	-	-	+	+	+	+	+	S+	S+	-
CAP36C2	nit1	XIII	-	-	-	-	-	S+	-	-	-	-	-	-
CAPDC1	nit1	XIII	-	-	-	-	-	+	-	-	-	-	-	-
CAPHC1	nit1	XIII	-	-	-	-	-	+	-	-	-	-	-	-
CAPIC3	nit1	XIII	-	-	-	-	-	S+	-	-	-	-	-	-
CAPWC1	nit1	XIII	-	-	-	-	-	S+	-	-	-	-	-	-
CAPYC1	nit1	XIII	-	-	-	-	-	S+	-	-	-	-	-	-
ORL1C5	nit1	XIII	-	-	-	-	S+	-	S+	S+	S+	-	S+	-
ORLIC7	nit1	XIII	-	-	-	-	-	-	-	-	-	-	-	-
ORP1C1	nit1	XIII	-	-	-	-	-	S+	-	-	-	-	-	-
ORMSC1	nit1	XIV	-	-	-	-	-	-	-	-	-	-	-	S+
IDP1C1	nit1	NC[3]	-	-	-	-	-	-	-	-	-	-	-	-
ORH1C1	nit1	NC	-	-	-	-	-	-	-	-	-	-	-	-

[a] Phenotypes based on growth on media containing nitrate, nitrite, and hypoxanthine. Mutants designated nit? had non-definative growth on nitrite medium and are presumed to be either nit1 or nit3 mutants.

[b] Complementation (+) was determined by the development of a dense zone of hyphal growth on MMT after 6 weeks. Pairings with "slow complementation" (S+) developed a faint zone of complementation after 6 weeks or a dense zone after 9 to 12 weeks. Pairings with slow, appressed growth after 12 weeks were labelled as negative (-).

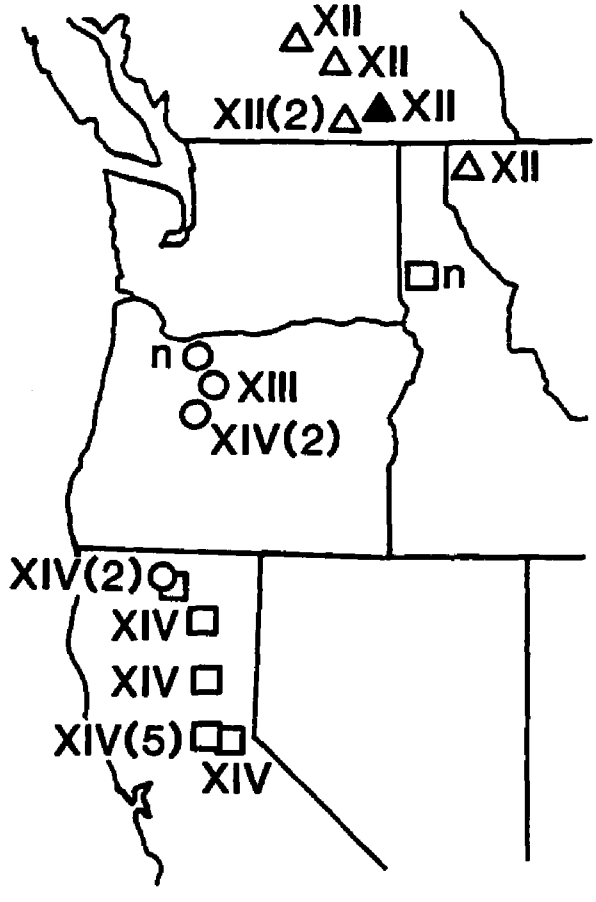

Figure 13. Geographic distribution of VC groups XII-XIV of *L. wageneri* var. *ponderosum*. Strains designated "n" were non-complementing. Symbols indicate electrophoretic types as follows: open triangles = electrophoretic type K, filled triangles = electrophoretic type L, open squares = electrophoretic type M, and open circles = electrophoretic type N. Unless otherwise indicated, each symbol represents one strain.

pairings were repeated.

Mutants from 28 of the 35 strains of var. *pseudotsugae* complemented at least one other mutant strain when paired. Ten VC groups were indicated (Tables 11, 12). Six VC groups contained a single, self-compatible strain, two others each contained two strains, and two VC groups (VC groups IV and VIII) each contained subgroups of two to five strains. Partial compatibility (slow positives) was found in some pairings between subgroups. There was, in addition, partial compatibility (slow complementation in one of two pairings) between mutant CAD19C2 of VC group VIIIb and mutant CAD18C1 of VC group IVb. Wild-type strains of these mutants were from the same geographic area, the Georgetown Divide area of the central Sierra Nevada, California.

All of the strains from which mutants were obtained that complemented well in this study were of electrophoretic type H or one of three electrophoretic types that each differed from type H for only one isozyme (Table 7). Strains of VC subgroup IVa were of electrophoretic types G or H, which differed for the enzyme esterase. Strains of VC subgroup IVb were of electrophoretic types H or I, with a difference at the enzyme β-glucosidase. The VC group VII contained the self-complementing strain IDD2 of electrophoretic type C. The other VC groups were comprised of strains of electrophoretic type H.

The geographic ranges of some of the vegetative compatibility groups of var. *pseudotsugae* overlapped in the central Sierra Nevada and the North Coast of California. Otherwise, the VC groups were geographically isolated (Fig. 14). Strains of VC group IV were from Vancouver Island, British Columbia, Washington, Oregon, and the north-central Sierra Nevada in California. Strains of VC groups VIII, IX, and X were

TABLE 11. COMPLEMENTATION AMONG NITRATE NON-UTILIZING MUTANTS OF VEGETATIVE COMPATIBILITY GROUPS (VC GROUPS) II - VII OF *LEPTOGRAPHIUM WAGENERI* VAR. *PSEUDOTSUGAE*.

Mutant	Phenotype[a]	VC group	BCD1C1 nitM II	BCD11C3 nitM III	CAD30C1 nitM IVa	WADUC3 nitM IVb	ORDQC1 nitM IVb	ORDPC3 nitM IVc	CAD19C2 nitM IVc	ORD1C1 nitM V	ORD2C4 nitM VI	IDD2C8 nitM VII
BCD1C2	nit1	II	+/-[b]	-/-	-/	-/	-/	-/	-/	-/	-/	-/-
BCD11C1	nit1	III	-/-	S+/S+	-/	-/	-/	-/	-/	-/	-/	-/-
BCD11C2	nit1	III	/-	/+	/-	/-	/-	/-	/-	/-	/-	/-
BCH1C3	nit1	IVa	-/	-/	+/	-/-	-/-	-/-	-/-	-/	-/	-/
BCDJC1	nit1	IVa	-/	-/	+/	-/-	S-/+	-/-	-/-	-/	-/	-/
CAD30C2	nit1	IVa	-/	-/	+/+	-/-	S+/+	-/S+	-/-	-/	-/	-/
WADUC2	nit1	IVb	-/	-/	-/-	+/+	+/	-/-	-/-	-/	-/	-/
ORD5C1	nit1	IVb	-/	-/	-/-	+/	+/	-/-	-/-	-/	-/	-/
ORDQC2	nit1	IVb	-/	-/	-/-	+/	+/+	-/-	-/-	-/	-/	-/
CAD32C1	nit1	IVb	-/	-/	-/S-	+/	+/	-/-	-/-	-/	-/	-/
ORDPC1	nit1	IVc	-/	-/	-/-	-/-	S+/-	+/+	+/	-/-	-/-	-/
ORD4C1	nit1	IVc	-/	-/	-/-	-/-	-/-	+/	+/	-/-	-/-	-/
CAD19C3	nit1	IVc	-/	-/	-/-	-/-	-/-	+/	+/+	-/-	-/-	-/
ORD1C3	nit1	V	-/	-/	-/	-/	-/	-/	-/	+/+	-/-	-/
ORD2C2	nit1	VI	-/	-/	-/	-/	-/	-/	-/	-/-	+/+	-/
IDD2C1	nit1	VII	-/-	-/-	-/	-/	-/	-/	-/	-/	-/	+/+

[a] Phenotypes based on growth on media containing nitrate, nitrite, and hypoxanthine.

[b] Complementation (+) was determined by the development of a dense zone of hyphal growth on MMT after 6 weeks. Pairings with "slow complementation" (S+) developed a faint zone of complementation after 6 weeks or a dense zone after 9 to 12 weeks. A pairing with slow complementation in which transfers from the zone of complementation resembled mutant growth was labelled S-. Pairings with slow, appressed growth after 12 weeks were labelled as negative (-). Slashes separate the results of two sets of pairings.

TABLE 12. COMPLEMENTATION AMONG NITRATE NON-UTILIZING MUTANTS OF VEGETATIVE COMPATIBILITY GROUPS (VC GROUPS) VIII - XI OF LEPTOGRAPHIUM WAGENERI VAR. PSEUDOTSUGAE.

Mutant	Phenotype[a]	VC group	CAD40C4 nitM VIIIa	CAD56C3 nitM VIIIa	CAD6C4 nitM VIIIb	CADFC1 nitM VIIIb	CAD31C18 nitM IX	CAD5C3 nitM X	CAD55C5 nitM X	CADXC2 nitM XI	CADXC5 nitM XI
CAD40C1	nit1	VIIIa	+/S+[b]	+/	S+/+	S+/S+-	/-	-/-	-/-	-/-	/-
CAD56C1	nit1	VIIIa	+/	+/S+	-?/+	-/S+	/-	-/-	-/-	-/-	/-
CAD6C1	nit1	VIIIb	-/-	-/-	+/+	+/	/-	-/-	-/-	-/-	/-
CADFC3	nit1	VIIIb	/S+	/-	/-	/+	/-	/-	/-	/-	/-
CAD1C2	nit1	VIIIb	S+/S+	S+/S+	+/	+/	/-	-/-	-/-	-/-	/-
CAD2C1	nit1	VIIIb	-/-	S+/S+	+/	+/	/-	-/-	-/-	-/-	/-
CAD27C1	nit1	VIIIb	S+/S+	S+/S+	+/	+/	/-	-/-	-/-	-/-	/-
CAD18C1	nit1	VIIIb	-/S+	-/-	+/	+/	/-	-/-	-/-	-/-	/-
CAD22C1	nit1	IX	/-	/-	/-	/-	/+	/-	/-	/-	/-
CAD22C3	nit1	IX	-/	-/	-/	-/	/+	-/	-/	-/	/-
CAD31C3	nit1	IX	/-	/-	/-	/-	/+	/-	/-	/-	/-
CAD31C4	nit1	IX	-/	-/	-/	-/	/+	-/	-/	-/	/-
CAD55C7	nit1	X	-/-	-/-	-/-	-/-	/-	+/	+/+	-/-	/-
CADXC1	nit1	XI	-/-	-/-	-/-	-/-	/-	-/-	-/-	S+/+	/+
CADXC3	nit1	XI	/-	/-	/-	/-	/-	/-	/-	/+	/+

[a] Phenotypes based on growth on media containing nitrate, nitrite, or hypoxanthine.

[b] Complementation (+) was determined by the development of a dense zone of hyphal growth on MMT after 6 weeks. Pairings with "slow complementation" (S+) developed a faint zone of complementation after 6 weeks or a dense zone after 9 to 12 weeks. Pairings with slow, appressed growth after 12 weeks were labelled as negative (-). Slashes separate the results of two sets of pairings.

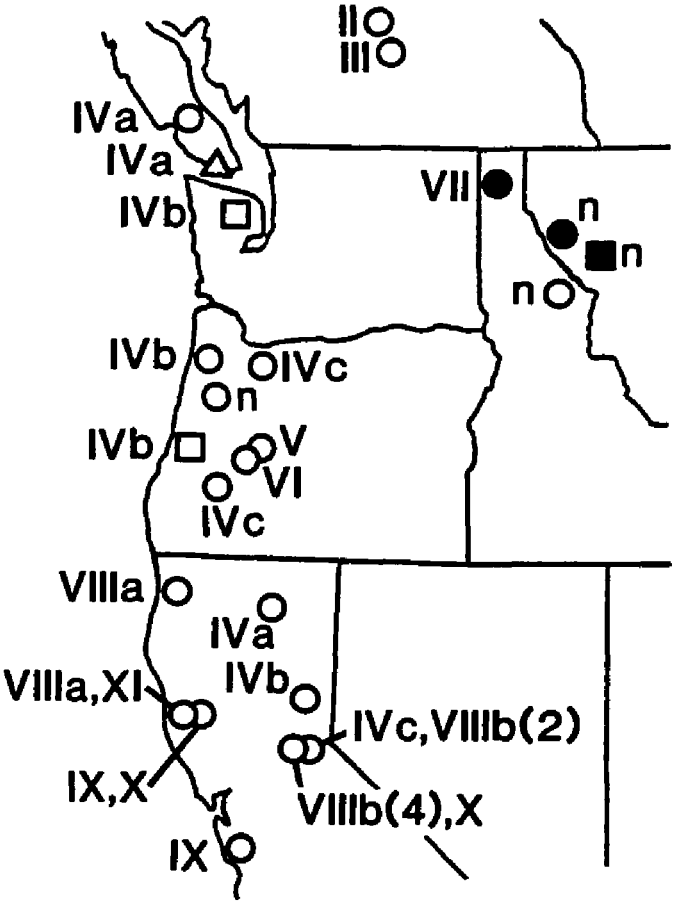

Figure 14. Geographic distribution of VC groups II-XI of *L. wageneri* var. *pseudotsugae*. Strains designated "n" were non-complementing. Symbols indicate electrophoretic types as follows: filled circles = electrophoretic type C, filled squares = electrophoretic type D, open triangles = electrophoretic type G, open circles = electrophoretic type H, and open squares = electrophoretic type I. Unless otherwise indicated, each symbol represents one strain.

only found in California. Strains of VC subgroup VIIIa and VC group IX were from the coastal ranges, but the six strains of VC subgroup VIIIb were from sites in the Sierra Nevada.

Mutants selected from seven wild-type strains of var. *pseudotsugae* failed to complement in pairings with nitM mutants from other strains (Table 7). These strains could not be tested for self-compatibility due to the recovery of only one mutant type. Five of the seven strains differed from the electrophoretic type H at from one to four enzymes. Six of the seven non-complementing strains were from the Rocky Mountains. Besides those shown in Fig. 14 (with the designation "n"), there were additional noncomplementing strains from Colorado and New Mexico (Table 7).

Two to four mutants from each major VC group of each variety that gave strong evidence of complementation in pairings within their VC group were selected for use in inter-variety pairings. In these pairings, nit1, nit3, and nitM mutants were paired in various combinations. The 150 pairings were all negative, indicating a lack of vegetative compatibility among the three varieties.

Discussion

Strains of *Leptographium wageneri* can anastamose to form heterokaryons, and when auxotrophic mutants with different phenotypes are paired, the heterokaryons may show genetic complementation. Heterokaryons only formed among mutants of the same variety, indicating genetic isolation between varieties. There was evidence of vegetative compatibility groups within var. *ponderosum* and var. *pseudotsugae*; members of a VC group had electrophoretic (isozyme) phenotypes that were similar or identical.

The estimate of seven mutant classes generated on chlorate media agrees with results obtained in other imperfect and ascomycetous fungi. Cove (23) obtained seven complementing classes of nitrate non-utilizing mutants in *Emericella nidulans*, and the same seven classes of mutants have been recovered in *Gibberella fujikuroi* (Sawada) Ito *apud* Ito & Kimura (anamorph, *Fusarium moniliforme* Sheldon) (53). Similarly, in *Fusarium oxysporum*, Correll *et al.* (20) recovered mutants of the three nitrogen metabolism phenotypes and estimated five or more classes of cofactor mutants. Four cofactor mutants were found in *Neurospora crassa* Shear & Dodge (59).

Complementation (usually slow complementation) was observed in some pairings of var. *ponderosum* among mutants with the nit1 phenotype. Some nit1 mutants may have been misidentified due to difficulties in determining nitrite utilization, but similar results have been reported from pairings between nit1 mutants in *Fusarium oxysporum* (20,32). As reviewed by Cove (23), nitrate reductase is composed of a number of subunits, and it is possible that nitrate reductase assembled from subunits having different defects could have at least partial enzymatic function.

Studies of anastamosis and heterokaryosis in other species of imperfect and ascomycete fungi reveal several ways in which heterokaryons can be maintained. In some species (e.g., *Aspergillus nidulans* (77), *Neurospora crassa* (75), and *Penicillium chrysogenum* Thom (78)), nuclei of both strains migrate and divide in the hyphae of the paired strains. Heterokaryons can become established in multinucleate hyphal tips, thus the heterokaryon can be maintained by further hyphal growth without recurrent anastamosis. In other species (e.g., *Verticillium*

dahliae Klebahn (83), *Magnaportha grisea* (Herbert) Barr (24), *Ophiostoma ulmi* (Buisman) Nannf. (13), and *Fusarium oxysporum* (80)), recurrent anastamosis is required, apparently because nuclei are not transferred from the cell in which anastamosis has occurred. Stable heterokaryons do not develop.

Hyphal tip cells of *L. wageneri* were invariably multinucleate in the examined strains, and the presence of enzyme electromorphs and nitrogen utilization phenotypes of both parents in some cultures derived from hyphal tips demonstrated that some hyphal tips are heterokaryotic. There were, however, indications that frequent anastamosis is important in maintaining heterokaryosis. There was only a low number of prototrophs in hyphal tip cultures derived from prototrophic colonies from complementing pairings of var. *pseudotsugae*, and no prototrophic hyphal tips were recovered from pairings of var. *wageneri* or var. *ponderosum*. Also, there was frequent sectoring of heterokaryotic, prototrophic cultures to auxotrophy. Thus it would appear that *L. wageneri* is capable of having multinucleate, heterokaryotic hyphal tips, but that under the experimental conditions, most hyphal tips become homokaryotic.

As with hyphal tips, stipe cells of developing conidiophores are multinucleate and can be heterokaryotic. Although conidia are uninucleate, both mutant types were identified among conidia of individual macronematous conidiophores, and the recovery of prototrophs in isolations from masses of conidia from single conidiophores was higher than from hyphal tips. There may be several explanations for the latter finding. First, stipe cells may have a greater number of conidia than do the individual cells of the hyphae that grow and divide

to form new hyphal tips. If cells are commonly heterokaryotic but nuclei of one genotype are rare, there may be a greater chance of detecting nuclei of different genotypes in cultures from masses of conidia than in cultures from hyphal tips. Alternatively, if heterokaryons are unstable and anastamosis is important in maintaining heterokaryosis, anastamoses are most abundant in the older portions of cultures where conidiophores form, whereas hyphal tips were from the advancing margin of subcultures where anastamosis is rare. The weak nitrate medium used for obtaining hyphal tips may also have affected the recovery of prototrophs, as this medium promotes thin, fast growth. Random loss of one or the other type of nucleus could easily occur under these growth conditions. In contrast, MMT is a medium more selective for prototrophy and growth of individual hyphae is much slower on this medium than on weak nitrate. Although not tested, heterokaryotic hyphal tips might be more abundant on this medium.

Low genetic diversity in *L. wageneri* was suggested by low isozyme diversity within varieties (73; Chapter 2), uniformity in electrophoretic types within geographic regions (Chapter 2), and the occurrence of relatively few vegetative compatibility groups. In other ascomycetous fungi, vegetative compatibility is determined by three to ten bi- or multi-factorial loci (2,8,9,13,52,65), and strains must be homoallelic at most of these loci for complementation to occur. If a similar basis for compatibility exists in *L. wageneri*, it would be expected that differences in the numbers of VC groups in the three varieties would correspond with relative differences in the amount of electrophoretic variation. As expected, var. *wageneri*, which had the lowest electrophoretic variation, had the fewest VC groups; var.

ponderosum was intermediate in both respects; and var. *pseudotsugae* had the greatest electrophoretic variation and the highest number of VC groups.

Compared to the number of electrophoretic types, there were fewer VC groups detected among strains of var. *wageneri* and var. *ponderosum*. However, in var. *pseudotsugae*, vegetative compatibility detected more variation than did enzyme electrophoresis; strains of the most common electrophoretic type (type H) were found in 9 VC groups (Table 7). The subgroups in VC groups IV and VIII of var. *pseudotsugae* may have differences at one or several VC loci. Also, many of the noncomplementing strains of var. *pseudotsugae* represented unique electrophoretic types and may represent additional VC groups. The lack of complementation in strain NES3 of var. *wageneri* and other strains could indicate low ability to anastamose and form heterokaryons, as has been reported in *F. oxysporum* (21,51).

The isozyme variation detected within VC groups or subgroups was minor and limited to a single isozyme difference, usually in the most variable of the enzymes assayed (Chapter 2). Jacobson and Gordon (51) and Elmer and Stevens (32) have similarly suggested that the pathogenic differences among strains of *F. oxysporum* f.sp. *melonis* and *F. oxysporum* f.sp. *asparagi*, respectively, that occur within VC groups may represent only minor genetic differences. A mixture of strains pathogenic to different hosts is also found in some VC groups of *Verticillium dahliae* (82). In contrast, Bosland and Williams (12) found complete correspondence between VC groups, electrophoretic types, and pathotypes of *Fusarium oxysporum* f.sp. *conglutinans*, and culture morphology and pathogenicity traits are homogeneous within VC groups of

F. oxysporum f.sp. *apii* (21) and *F. oxysporum* f.sp. *cubense* (76).

The results of vegetative compatibility testing and enzyme electrophoresis suggest that reproduction in *L. wageneri* is largely or solely asexual, although a teleomorph *(Ophiostoma wageneri* (Goheen & Cobb) Harrington) has been described (36). The limited number of VC groups in *Leptographium wageneri* contrasts sharply with the extensive diversity found in the related Ascomycete, *Ophiostoma ulmi* (13). In an extensive study of vegetative compatibility in the races and strains of *O. ulmi*, Brasier (13) reported that in one sample of the endemic population (i.e., the "non-agressive strain") in England, nearly every isolate was a member of a different VC group. Similarly high numbers of VC groups are present in local, sexual populations of *Neurospora crassa* (59) and North American populations of *Cryphonectria parasitica* (Murrill) M.E. Barr (3). Sexual recombination among numerous compatibility loci accounts for the predominance of strains with differences in compatibility. Anagnostakis (2) has recovered 106 combinations of VC alleles in just one cross between strains of *C. parasitica*.

In contrast, in *Leucostoma kunzei* (Fr.) Munk ex Kern (cause of cytospora canker of spruce) (79) and *C. parasitica* in Europe (54), perithecia are infrequently produced and all isolates from a given group of trees may be in the same VC group. Relatively few VC groups are also found in strictly asexual fungi (10,12,21,51,76,82). The number of VC groups would tend to be low in a species that lacks an effective method of generating new allelic combinations.

Asexuality, founder effects, and clonal selection could explain the establishment and maintenence of homogeneity within local populations of *L. wageneri*. Crow and Kimura (25) have suggested that in

organisms with strictly asexual reproduction, the genome, rather than the allele, is the unit of selection. Hypothetically, a mixture of *L. wageneri* strains could be introduced into a tree by bark beetle attack. If one VC group predominates, a situation analogous to experimental data in wood-decay Basidiomycetes (84) may occur: strains of rarer VC groups may be unable to compete effectively for substrate beyond the site of introduction, whereas strains of the predominant VC group could continue to spread throughout the root system and to adjacent trees. Infection centers that thus develop might then be genetically uniform.

The localized occurrence of VC groups of *Cryptostroma corticale* (Ellis & Everhart) Gregory & Waller (an asexual pathogen of sycamores) (10), of some of the 16 VC groups of *Verticillium dahliae* (82), and of some of the VC groups of *Fusarium oxysporum* f.sp. *cubense* (76) suggests clonal and frequency-dependant selection (selection against rare VC groups) as a potential model for explaining population structure of some asexual plant pathogens.

The presence of more than one electrophoretic variant within some VC groups of *L. wageneri*, the recovery of mixed conidia from heterokaryotic conidiophores, and the recovery (albeit rare) of heterokaryotic hyphal tips suggest the possibility that heterokaryosis could play at least a limited role in maintaining diversity within VC groups or in generating new strains through parasexual recombination. The parasexual process was not explored in this study, and no attempts were made to characterize prototrophs as heterokaryons vs. diploid strains, i.e., strains that could give rise to parasexual recombinants. Thus, the lack of recombinants among the 109 hyphal tip cultures tested for non-parental combinations of nitrogen metabolism and isozyme phenotypes

does not rule out the possibility of parasexual recombination in *L. wageneri*.

Nonetheless, genetic variation in *L. wageneri* appears to be extremely limited. The relatively low number of VC groups per variety, the geographic distribution of strains in each VC group, and the correlation between electrophoretic type and VC group found in *Leptographium wageneri* are indications that this fungus is essentially asexual. Genetic diversity may be further constrained by the lack of inter-variety heterokaryosis and the limits on intra-variety heterokaryosis imposed by vegetative incompatibility. Clonal selection and founder effects are suggested as being of importance in determining population structure in this and other asexual fungi.

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